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SARS-CoV-2 specific memory T cell epitopes identified in COVID-19-recovered subjects

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ABSTRACT

The COVID-19 pandemic caused by SARS-CoV-2 infection poses a serious threat to public health. An explicit investigation of COVID-19 immune responses, particularly the host immunity in recovered subjects, will lay a foundation for the rational design of therapeutics and/or vaccines against future coronaviral outbreaks. Here, we examined virus-specific T cell responses and identified T cell epitopes using peptides spanning SARS-CoV-2 structural proteins. These peptides were used to stimulate peripheral blood mononuclear cells (PBMCs) derived from COVID-19-recovered subjects, followed by an analysis of IFN- γ -secreting T cells by enzyme-linked immunosorbent spot (ELISpot). We also evaluated virus-specific CD4 or CD8 T cell activation by flow cytometry assay. By screening 52 matrix pools (comprised of 315 peptides) of the spike (S) glycoprotein and 21 matrix pools (comprised of 102 peptides) spanning the nucleocapsid (N) protein, we identified 28 peptides from S protein and 5 peptides from N protein as immunodominant epitopes. The immunogenicity of these epitopes was confirmed by a second ELISpot using single peptide stimulation in memory T cells, and they were mapped by HLA restrictions. Notably, SARS-CoV-2 specific T cell responses positively correlated with B cell IgG and neutralizing antibody responses to the receptor-binding domain (RBD) of the S protein. Our results demonstrate that defined levels of SARS-CoV-2 specific T cell responses are generated in some, but not all, COVID-19-recovered subjects, fostering hope for the protection of a proportion of COVID-19-exposed individuals against reinfection. These results also suggest that these virus-specific T cell responses may induce protective immunity in unexposed individuals upon vaccination, using vaccines generated based on the immune epitopes identified in this study. However, SARS-CoV-2 S and N peptides are not potentially immunogenic, and none of the single peptides could universally induce robust T cell responses, suggesting the necessity of using a multi-epitope strategy for COVID-19 vaccine design.

1. Introduction

The COVID-19 pandemic caused by SARS-CoV-2 infection poses a serious threat to global public health. As of June 15, 2021, there have been 175,847,347 confirmed cases of COVID-19 and 3807,276 deaths reported worldwide - with 33,140,498 confirmed cases and 594,644 deaths in the United States alone (World Health Organization, 2021).

Notably, the majority of COVID-19 patients spontaneously resolve the infection, suggesting that host immunity is naturally induced in COVID-19 patients. Emerging data have revealed important insights into host immune responses, particularly the adaptive T cell response, in patients who had mild versus severe SARS-CoV-2 infection (Yu et al., 2020; Arunachalam et al., 2020; Zhang et al., 2020; Chen and John Wherry, 2020). However, it remains unclear whether T cell responses to

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SARS-CoV-2 play a role in the disease progression, viral clearance, or protection. Additionally, the specific viral epitopes that induce T cell responses have not yet been identified.

Recent studies on SARS-CoV-2 immune responses have shown that multiple SARS-CoV-2 proteins could induce virus-specific T cell responses in SARS-CoV-2-infected individuals (Zhang et al., 2020; Sattler et al., 2020; A Grifoni et al., 2020). However, precise screening and identification of T cell immune epitopes using SARS-CoV-2 major structural protein peptides to stimulate memory T cells derived from COVID-19-recovered subjects for a recall response are lacking. Further analysis of these virus-specific T cell responses in COVID-19-recovered subjects may provide important information regarding the host adaptive immunity against SARS-CoV-2 infection. Also, a major obstacle to developing safe and effective therapeutics and/or vaccines against SARS-CoV-2 is the lack of knowledge regarding the specific viral epitopes that are recognized by the human immune system. Therefore, identification of these immune epitopes may foster the rational design of therapeutics and/or vaccines against future outbreaks.

While major T cell epitopes in both spike (S) and nucleocapsid (N) proteins of SARS-CoV-2 have been predicted using bioinformatics approaches (Kiyotani et al., 2020; Grifoni et al., 2020; Ranga et al., 2020), these epitopes need to be experimentally validated using clinical samples from COVID-19-recovered patients in “real world”. In this study, we characterized SARS-CoV-2 specific T cell responses and identified T cell immune epitopes using 52 S-matrix pools (comprised of 315 peptides) and 21 N-matrix pools (comprised of 102 peptides) to stimulate peripheral blood mononuclear cells (PBMCs) derived from patients who had recovered from SARS-CoV-2 infection, followed by measuring IFN- γ -secreting T cells using an enzyme-linked immunosorbent spot (ELISpot) assay. Additionally, we evaluated virus-specific CD4 or CD8 T cell activation by flow cytometry analysis. Our study provides insights into the immunogenicity of SARS-CoV-2 and, importantly, identifies the immunodominant T cell epitopes that may be responsible for the viral clearance and/or protection. These results will assist in SARS-CoV-2 vaccine design and facilitate the evaluation of vaccine effectiveness. Several of these identified epitopes are promising candidates for developing therapeutic agents against SARS-CoV-2 infection.

2. Materials and methods

2.1. Subjects

The study subjects were composed of two groups: 11 mild to moderate, non-hospitalized COVID-19-recovered patients and 4 control subjects, including 2 healthy subjects (HS) and 2 Influenza (Flu) patients. All COVID-19 patients were diagnosed using a positive PCR nucleic acid test and had recovered at least 2 weeks after the diagnosis. Blood from HS was obtained from BioIVT (Gray, TN) and was free of HBV, HCV, and HIV infections. The healthy control samples and Flu samples were confirmed by a negative serology test. PBMCs were isolated from 30-ml of blood by Ficoll density centrifugation (GE

Healthcare; Piscataway, NJ) and stored in liquid nitrogen until used. The characteristics of the COVID-19 subjects are shown in Table 1.

2.2. ELISpot assay

The SARS-CoV-2 ELISpot assay was performed using ELISpot^{PRO} 96-well plates (Mabtech, Nacka Strand, Sweden) following the manufacturer's guidelines and published protocols (Janetzki et al., 2015). Briefly, PBMCs were thawed and rested in 50-ml Corning bioreactor tubes overnight. For each subject, 100 μ l of 2×10^6 PBMCs/ml cell suspension (2×10^5 PBMCs/well) were stimulated overnight (~16 h) with 100 μ l of SARS-CoV-2 matrix peptide pools (2 μ g/ml of each peptide; JPT, Germany), SARS-CoV-2 individual peptides (2 μ g/ml; JPT), mAb CD3-2 (1:500 dilution; Mabtech), CMV peptide pool control (2 μ g/ml; Mabtech), or blank control (complete RPMI 1640 medium with DMSO). The numbers of SARS-CoV-2-specific IFN- γ -secreting T cells/ 2×10^5 PBMCs, referred to as IFN- γ spot forming cells (SFCs), were determined using an AID iSpot Reader (AID, Strasberg, Germany) and analyzed using ELISpot 7.0 Software (AID). Wells containing SFC numbers greater than mean + 3SD of the blank controls were considered positive. A dotted line is drawn in each figure to show the positive threshold.

2.3. Epitope mapping peptide set (EMPS)

A set of 52 Matrix Pools (MPs) and 315 individual overlapping peptides spanning the whole Spike Glycoprotein and a set of 21 MPs and 102 individual overlapping peptides spanning the whole Nucleoprotein of SARS-CoV-2 for epitope identification and mapping were purchased from JPT (Berlin, Germany). The mapping layouts are shown in Tables 2 and 3. In this layout, the MP design arranges each peptide in a square matrix, and MPs comprise peptides from a single row or a single column. According to this layout, each peptide is present in two MPs. The tested MPs displaying cell stimulation pinpoints the specific peptide in the intersection on the layout.

2.4. Flow cytometric analysis

Based on our ELISpot results, PBMCs were cultured overnight (~16 h) in the presence of positive MPs or single peptides at a concentration of 1 μ g/mL in 96-well U-bottom plates. For CD69 activation marker staining, the cells were harvested and stained with anti-CD3-PerCP, anti-CD4-FITC, anti-CD8-FITC, or anti-CD69-PE antibodies (Biolegend, San Diego, CA) at room temperature for 30 min and then subjected to flow cytometry analysis. For intracellular cytokine staining (ICS), the assay was performed as previously described (Zhao et al., 2019). Briefly, Brefeldin A was added 4 h before harvesting. The cells were harvested and stained with anti-CD3-PerCP, anti-CD4-FITC, or anti-CD8a-FITC antibodies as described above. Then, the cells were washed, fixed, and permeabilized with the Foxp3 Transcription Factor Staining Buffer Set (Invitrogen, Carlsbad, CA), followed by staining with anti-IFN- γ -PE

Table 1
Characteristics of COVID-19 patients.

ID	Age	Gender	Symptom (Self-assessment)	Hospitalized (Y/N)	Sampling (Day)	Past Medical History
P1	54	F	Mild	N	74	N/A
P2	54	M	Mild	N	72	N/A
P3	51	F	Mild	N	78	N/A
P4	47	F	Moderate	N	75	Asthma
P5	20	F	Mild	N	77	N/A
P6	30	M	Moderate	N	88	N/A
P7	46	F	Moderate	N	119	Cardiomyopathy
P8	33	M	Mild	N	24	Hypertension, HIV
P9	42	F	Moderate	N	29	N/A
P10	42	M	Moderate	N	32	N/A
P16	47	M	Mild	N	15	Hypertension

Table 2
SARS-CoV-2 S-Matrix Pools for mapping specific T cell epitopes.

MPs	1	2	3	4	5	6	7	8	9	10	11	12	13
14	1	2	3	4	5	6	7	8	9	10	11	12	13
15	14	15	16	17	18	19	20	21	22	23	24	25	26
16	27	28	29	30	31	32	33	34	35	36	37	38	39
17	40	41	42	43	44	45	46	47	48	49	50	51	52
18	53	54	55	56	57	58	59	60	61	62	63	64	65
19	66	67	68	69	70	71	72	73	74	75	76	77	78
20	79	80	81	82	83	84	85	86	87	88	89	90	91
21	92	93	94	95	96	97	98	99	100	101	102	103	104
22	105	106	107	108	109	110	111	112	113	114	115	116	117
23	118	119	120	121	122	123	124	125	126	127	128	129	130
24	131	132	133	134	135	136	137	138	139	140	141	142	143
25	144	145	146	147	148	149	150	151	152	153	154	155	156
26	157	158											

MPs	27	28	29	30	31	32	33	34	35	36	37	38	39
40	159	160	161	162	163	164	165	166	167	168	169	170	171
41	172	173	174	175	176	177	178	179	180	181	182	183	184
42	185	186	187	188	189	190	191	192	193	194	195	196	197
43	198	199	200	201	202	203	204	205	206	207	208	209	210
44	211	212	213	214	215	216	217	218	219	220	221	222	223
45	224	225	226	227	228	229	230	231	232	233	234	235	236
46	237	238	239	240	241	242	243	244	245	246	247	248	249
47	250	251	252	253	254	255	256	257	258	259	260	261	262
48	263	264	265	266	267	268	269	270	271	272	273	274	275
49	276	277	278	279	280	281	282	283	284	285	286	287	288
50	289	290	291	292	293	294	295	296	297	298	299	300	301
51	302	303	304	305	306	307	308	309	310	311	312	313	314
52	315												

Table 3
SARS-CoV-2 N-Matrix Pools for mapping specific T cell epitopes.

MPs	1	2	3	4	5	6	7	8	9	10
11	1	2	3	4	5	6	7	8	9	10
12	11	12	13	14	15	16	17	18	19	20
13	21	22	23	24	25	26	27	28	29	30
14	31	32	33	34	35	36	37	38	39	40
15	41	42	43	44	45	46	47	48	49	50
16	51	52	53	54	55	56	57	58	59	60
17	61	62	63	64	65	66	67	68	69	70
18	71	72	73	74	75	76	77	78	79	80
19	81	82	83	84	85	86	87	88	89	90
20	91	92	93	94	95	96	97	98	99	100
21	101	102								

antibody (Biolegend) for 45 min at room temperature. Approximately 200,000 events were collected for flow cytometry analysis. For the activation-induced marker (AIM) assay, the cells were stained with anti-CD154-FITC, anti-CD4-PE, anti-CD8-PerCP, and anti-CD137-APC antibodies. Controls for these assays included isotype control antibodies, single staining, and unstained cells, which were used for gating and compensation. Samples were analyzed by a BD AccuriC6 Plus flow cytometer and FlowJo V10 software.

2.5. Detection of SARS-CoV-2 specific IgG and neutralization antibodies

High-sensitivity SARS-CoV-2 S1 IgG Quantitative ELISA kit (BioVendor, Asheville, NC) and SARS-CoV-2 Surrogate Virus Neutralization Test kit (GenScript) were used to determine the concentration of S1 IgG and Neutralization antibody levels respectively in the COVID-19 convalescent plasma as described previously (Wang et al., 2021).

2.6. Statistics

The data were analyzed using Prism 7 software and are presented as mean \pm SEM. The outliers were identified by the ROUT method ($Q = 1.000\%$) and excluded from the analysis. Unpaired t-tests were used to compare means of two independent groups with equal variances, and Welch's correction was utilized if unequal variances were found. Comparisons between two groups with skewed data were analyzed using the nonparametric Mann-Whitney U test. Correlations between anti-S RBD IgG or antibody neutralization rates and SFCs were analyzed by Spearman's correlation. Details are noted in the figure legends.

3. Results

3.1. Identification of T cell epitopes using overlapping peptides spanning SARS-CoV-2 S protein

The SARS-CoV-2 S protein is regarded as a leading target in vaccine development (Poh et al., 2020; Samrat et al., 2020; Pillay, 2020). Previous studies reported the identification of putative T cell epitopes using *in silico* tools (Crooke et al., 2020; Abraham Peele et al., 2020; Jakhar et al., 2020; Zaheer et al., 2020) and evaluated T cell responses using COVID-19 patients' immune cells stimulated with SARS-CoV-2 S protein or mixed peptides (rather than discrete, individual peptide motifs) (Grifoni et al., 2020; Braun et al., 2020). Thus, we set out to identify virus-specific T cell epitopes using overlapping peptide stimulation in memory T cells from COVID-19-recovered patients, followed by ELISpot assay. To this end, we employed the JPT peptide Matrix Pools (MPs), which enables fast mapping of T-cell epitopes while utilizing minimal amounts of patient cells. The MPs consist of multiple 15mers peptides with 11-overlapping amino acids in each peptide, spanning the whole sequence of the SARS-CoV-2 S-protein in such a way that T cell stimulation is optimized while the chance of missing T cell epitopes is minimized (PepMix concept). The 315 SARS-CoV-2 S-peptides were pooled according to a matrix design that generates 52 subgroups (Table 2). Representative ELISpot images revealed that while the control subjects HS1 and Flu2 were nonresponsive, COVID-19 patients (P1 and P2) exhibited a positive response to SARS-CoV-2 S-MP-43 peptide stimulation (Fig. 1A). All control subjects (HS1, HS2, Flu1, and Flu2) showed no response to SARS-CoV-2 S-MPs (Fig. 1B-E), whereas all COVID-19-recovered patients (P1-P5) showed positive T cell responses after stimulation (Fig. 1F-IJ).

It is worth noting that there was a large variation in the response levels as well as the number of peptides recognized by memory T cells among different patients. Amongst the five tested COVID-19-recovered patients, P2 exhibited the strongest IFN- γ response, including the highest number of spot-forming cells per well (SFCs/well) and elicited responses to more peptides (38/52 MP), whereas P5 showed the poorest response (lowest SFCs/well) and elicited a response to only 5/52 MPs.

Fig. 1K illustrates summary data of SFCs for P1-P5, based on which we identified virus-specific epitopes by pinpointing the intersection on the mapping layout shown in Table 2. For SARS-CoV-2 S protein, 315 peptides were pooled into 52 MPs according to the matrix design. Those MPs with positive reactions are shaded in pink, and individual peptides (highlighted in red) are pinpointed as responsible for the positive T cell response. Using this strategy, we identified 28 candidate peptides in SARS-CoV-2 S-MPs as S antigen-specific T cell epitopes. The 28 candidate peptides were then confirmed individually in a second ELISpot assay.

In addition to the IFN- γ ELISpot assay, we utilized intracellular immunostaining to confirm the levels of IFN- γ^+ producing CD4 or CD8 T cells after stimulation with S-MPs. Fig. 1L shows the flow cytometry gating strategy used to detect S-specific T cells after *in vitro* stimulation with the SARS-CoV-2 S-MPs, which showed a positive response in ELISpot assay. The representative plots show IFN- γ expression in CD4 and CD8 T cells from a COVID-19 patient (P2) and a control subject (Flu2). While flow cytometry appeared to be less sensitive than the ELISpot assay, CD4 and CD8 T cells from patient P2 exhibited an increase in virus-specific IFN- γ expression compared to the control subject Flu2. Fig. 1M summarizes percentages of IFN- γ^+ cells within CD4 or CD8 T cells. The results revealed that COVID-19-recovered patients exhibited virus-specific CD4 T cell responses, as evidenced by the increases in IFN- γ producing cells in response to stimulation with SARS-CoV-2 S-MPs. Therefore, we summarized the percentage of IFN- γ -producing CD4 and CD8 T cells based on their frequencies in the peripheral blood. Fig. 1N shows that both IFN- γ^+ CD4 $^+$ and IFN- γ^+ CD8 $^+$ T cells from COVID-19-recovered patients were expanded in response to SARS-CoV-2 S-MPs compared to the controls, providing further evidence that virus-specific T cell responses are elicited in COVID-19-recovered patients.

To further characterize SARS-CoV-2 specific T cell epitopes, we mapped the 28 candidate peptides by a second ELISpot assay using a single peptide stimulation. As shown in Fig. 2A-2M, the control subjects (HS1, HS2, and Flu2) showed no response to SARS-CoV-2 S candidate peptide stimulations (Fig. 2A-2B, 2D), whereas the control Flu1 exhibited a marginally positive response to SARS-CoV-2 S-89 and S-203 peptide stimulations (Fig. 2C). Amongst the nine COVID-19 patients tested, only P2, P4, P6, P8, P9, and P16 showed positive T cell responses to at least one S single peptide (Fig. 2F-2H, 2J, 2K, and 2M). Notably, P2, P6, P8, and P16 are male patients. Patient P8 elicited the strongest IFN- γ response, including the highest level of IFN- γ production (SFCs/well) and the most responses to the candidate peptides (8/28 MP), whereas patients P4 and P9 showed the poorest responses (1/28 MP). The other three COVID-19 patients (P1, P7, and P10) did not show a positive response to any single S peptide stimulation (Fig. 2E, 2I, and 2L). Fig. 2N summarizes the mean SFCs from the nine COVID-19-recovered patients in response to 28 S single peptides, of which we identified four virus-specific immunodominant epitopes (S42, S50, S80, and S81).

Flow cytometry analysis of activation-induced marker (AIM) is a cytokine-independent assay designed to test antigen-specific T cell activation and is widely used to identify virus- or vaccine-specific T cell responses (Braun et al., 2020; Jiang et al., 2019; Mateus et al., 2020; Dan et al., 2016). Recent studies using the AIM assay (Grifoni et al., 2020; Mateus et al., 2020) revealed increased T cell activation in COVID-19 patients compared to unexposed subjects. To examine the generation of SARS-CoV-2 specific CD4 and CD8 T cells after peptide stimulation, we utilized the AIM assay to measure virus-specific T cell activation in COVID-19-recovered patients. Specifically, we stimulated PBMCs from ten COVID-19-recovered patients and two control subjects (one HS and one flu) with 28 SARS-CoV-2 S single peptides as indicated. We used the CMV peptide pool and anti-CD3 mAb as positive controls and DMSO as a negative control. CD40L $^+$ and CD40L $^+$ 4-1BB $^+$ were used to determine the activation of SARS-CoV-2 specific CD4 T cells, whereas 4-1BB $^+$ and CD40L $^+$ 4-1BB $^+$ were used to determine the activation of SARS-CoV-2 CD8 T cells. Fig. 2O shows the flow cytometry gating

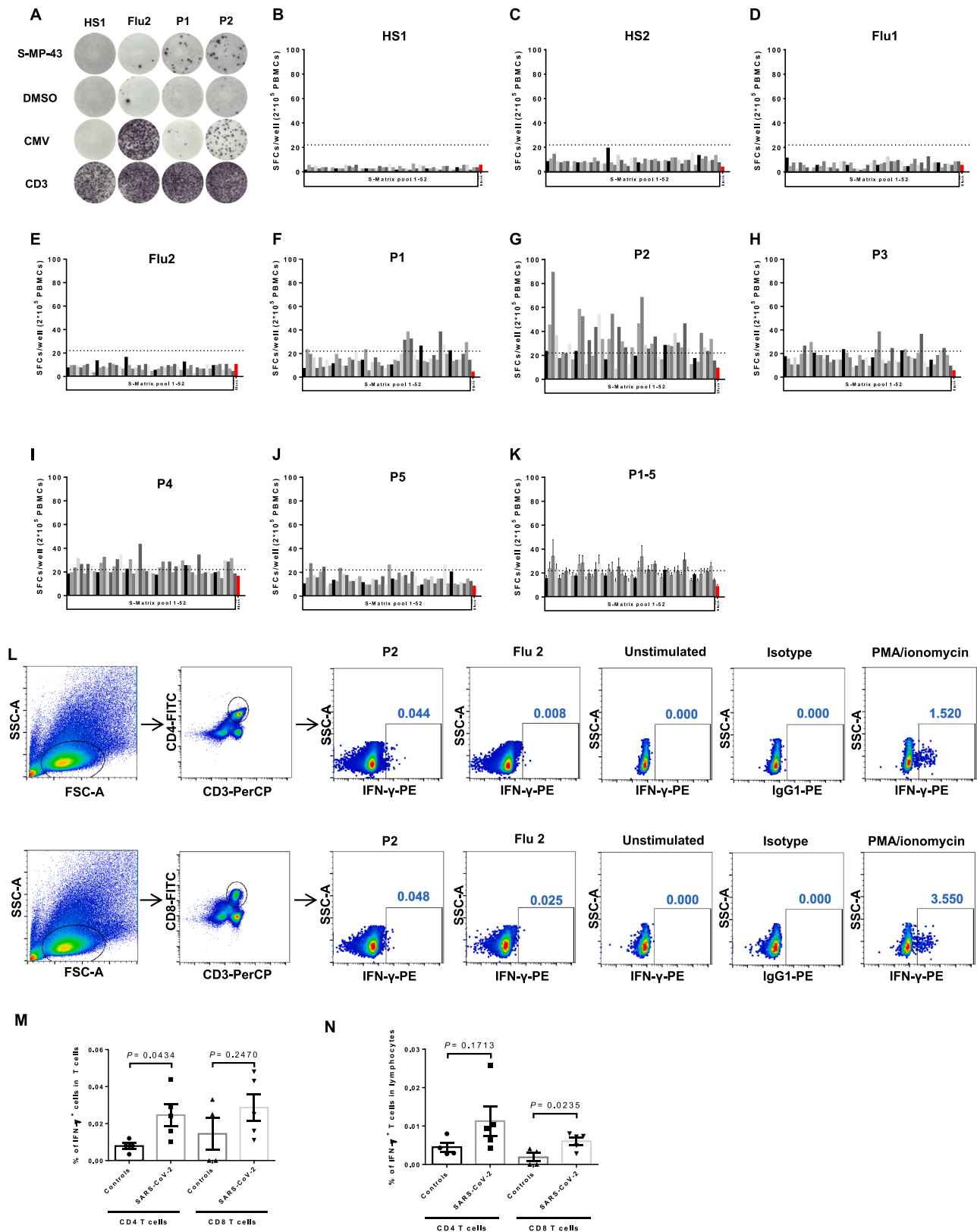


Fig. 1. Detection of SARS-CoV-2 specific T cell response to the S peptide pools in COVID-19-recovered patients by IFN- γ ELISpot and flow cytometry. A) Representative results from the ELISpot assay of PBMCs from HS1, Flu2, P1, and P2 stimulated with S-MP-43 peptide, DMSO, CMV peptide pools, or CD3-2 mAb. B-J) Quantification of SFCs from the ELISpot assay of PBMCs derived from four controls (2 HS and 2 Flu patients) and five COVID-19-recovered patients. The dotted line (mean+3SD=22 of all negative control wells) represents the threshold for a positive response. Blank control is shown in red. K) Summary data (mean \pm SEM) of SFCs derived from P1-P5. L) Representative pseudocolor plots of flow cytometry analysis and gating strategy for lymphocytes, CD4 T cells, CD8 T cells, and IFN- γ in gated CD4 and CD8 T cells. M) Frequency (%) of IFN- γ ⁺ CD4 T cells and CD8 T cells detected by flow cytometry. N) Frequencies (%) of IFN- γ ⁺ CD4⁺ T cells and IFN-

γ^+CD8^+ T cells within lymphocytes detected by flow cytometry. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

strategy for the AIM assay. Fig. 2P and Fig. 2Q show the percentages of CD40L⁺ and CD40L⁺4-1BB⁺ cells within the CD4 T cell population. The results demonstrated that 13 out of 28 (13/28) peptides and 6/28 peptides, respectively, could induce an increase in CD40L⁺ and CD40L⁺4-1BB⁺ CD4 T cells. Fig. 2R and Fig. 2S show the percentages of 4-1BB⁺ and CD40L⁺4-1BB⁺ cells within the CD8 T cell population. These results reveal that 9/28 peptides and 13/28 peptides can increase 4-1BB⁺ and CD40L⁺4-1BB⁺ cells within CD8 T cells. Of note, peptides S80 and S81 were identified as immunodominant epitopes in both ELISpot and AIM assays.

3.2. Identification of T cell epitopes using overlapping peptides spanning SARS-CoV-2 N protein

The SARS-CoV-2 S protein displays high rates of mutations (i.e., quasispecies), which can drive its resistance to neutralization antibodies and T cell responses, or the lack of protection to prophylactic vaccines (Korber et al., 2020). In contrast, the SARS-CoV-2 N protein is relatively conserved and stable, with 90% amino acid homology among various strains, and accumulates fewer mutations over time (Grifoni et al., 2020). The N proteins of many coronaviruses are highly immunogenic and expressed abundantly during viral infection (Cong et al., 2020). An elevated N protein-induced IFN- γ production has been observed in COVID-19-recovered patients (Thijssen et al., 2020), indicating that SARS-CoV-2 N protein might serve as a good candidate for vaccine development (Ahlen et al., 2020). To evaluate T cell responses and identify T cell epitopes within the N protein, PBMCs derived from COVID-19-recovered patients were stimulated with SARS-CoV-2 N-MPs (21 MPs comprised of 102 peptides), and IFN- γ production was measured by ELISpot assay. As shown in Fig. 3A, patients P2 and P3 showed positive responses to N-MP-19, whereas patients P1, P4, and P5 did not elicit responses, similar to the unstimulated cells (< 20 SFCs/well). Patients P2, P4, and P5 exhibited strong T cell responses to the CMV peptide pool, and all patients had robust responses to CD3 mAb stimulation. The control subjects exhibited negative reactions to stimulation with all of the peptides (Fig. 3B-3E), whereas four of five COVID-19 patients showed positive responses to stimulation with at least one N-MP (Fig. 3F-3J). Similar to our findings in S-MP-stimulated T cell responses (Fig. 1), we observed a large variation in the SARS-CoV-2 N-induced T cell responses among different COVID-19 patients, although their blood samples were collected within the same time frame (72–78 days post-infection). Among the five COVID-19-recovered patients, both P2 and P3 exhibited stronger IFN- γ responses (numbers of SFCs/well) as well as higher numbers of reactions to MPs (10/21 MPs and 12/21 MPs). On the other hand, P1, P4, and P5 elicited minimal responses. Notably, P2 showed the highest T cell reactivity (320 SFCs/well) against N-MP19 stimulation. Fig. 3K shows the mean \pm SEM of SFCs/well for P1-P5, of which we identified five SARS-CoV-2 N-specific T cell epitopes using the same MP pinpoint principle as explained above (Table 3).

To confirm the ELISpot assay results, we examined SARS-CoV-2 specific T cell response to N-MP19 using flow cytometry to measure the T cell early activation marker CD69. Following stimulation with N-MP 19, the PBMCs were harvested and stained for CD3, CD4, CD8, and CD69 antibodies. As shown in Fig. 3L, patient P2 exhibited a higher frequency of CD69⁺ cells within both CD4 and CD8 T cell populations compared to the control Flu2. The mean frequency of CD69⁺ cells in SARS-CoV-2 and control groups revealed that CD69 expression was elevated in both CD4 and CD8 T cell populations (Fig. 3M) and also in the overall lymphocyte population (Fig. 3N; adjusted by CD4 and CD8 T cell frequencies) from COVID-19-recovered patients compared to the control subjects.

Moreover, these five N antigen-specific T cell epitopes were confirmed by a second ELISpot assay, using each individual peptide stimulation in the PBMCs. The representative results in Fig. 4A show that the control Flu2 subject did not respond to SARS-CoV-2 N81 and N-88 but elicited a strong response to both CMV peptide and CD3 mAb stimulation. Of note, patient P1 only responded to anti-CD3 mAb, whereas patient P2 exhibited positive responses to peptides N-81 and N-88 as well as CMV peptides and CD3 mAb stimulation (positive controls). The controls (HS1 and HS2 in Fig. 4B-4C; Flu1 and Flu2 in Fig. 4D-4E) did not respond to SARS-CoV-2 N single peptide stimulation. Fig. 4F-4P shows the virus-specific T cell responses to a single N peptide in each COVID-19-recovered patient. Four out of five candidate peptides were confirmed to induce positive responses in at least one COVID-19 subject.

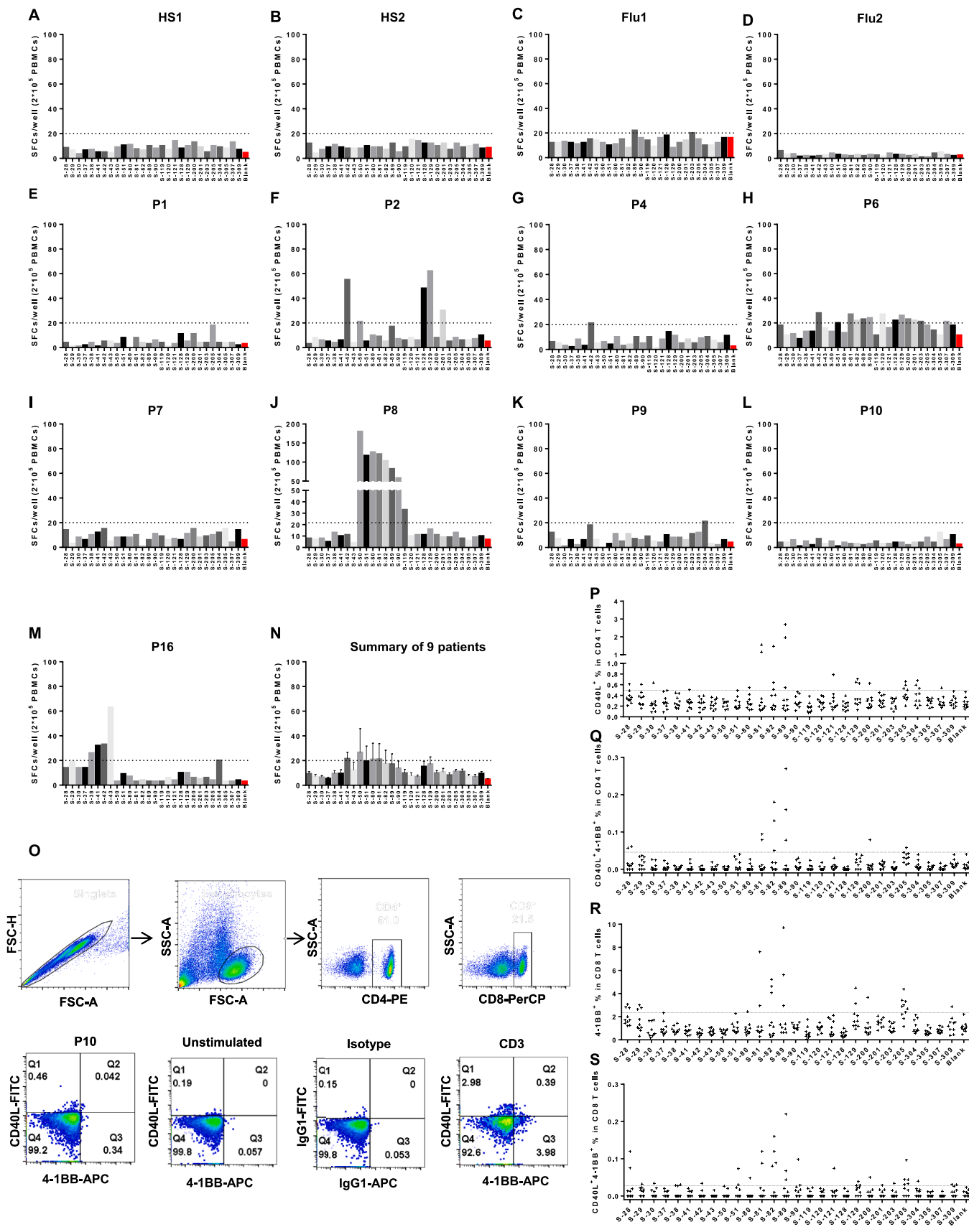
Additionally, the AIM assay was used to identify T cell activation and virus-specific epitopes. The results demonstrated that N-81 and N-85 could induce increases in CD40L expression in CD4 T cells (Fig. 4Q), while N-81 and N-88 could induce co-expression of CD40L and 4-1BB in CD4 T cells (Fig. 4R). Similarly, only peptide N-85 increased the expression of 4-1BB in CD8 T cells (Fig. 4S), whereas, individual peptides N-81, N-85, and N-88 could induce CD40L and 4-1BB co-expression in CD8 T cells from 1 or 2 out of 10 COVID-19 patients (Fig. 4T). Taken together, these results demonstrate that peptides N-81, N-82, N-85, and N-88 are potential T cell immune epitopes that can induce T cell recall responses in some COVID-19-recovered patients.

3.3. Prediction of HLA restrictions of SARS-CoV-2-specific T cell epitopes

We determined the putative MHC class I and II restrictions of the identified epitopes based on NetMHCpan-4.1 and NetMHCIIpan-4.0 (Reynisson et al., 2020) - two web servers that predict bindings between peptides and MHC-I or MHC-II. The Allele Frequency Net Database (Gonzalez-Galarza et al., 2020) was used to identify HLA alleles expressed in the U.S. Caucasian population. Based on these databases and our research subjects' race, we assigned HLA-A, HLA-B, and HLA-DRB1 restrictions for the epitope binding prediction (a total of 49 alleles, listed in Table 4), which reflect the major HLA alleles in the U.S. Caucasian population. A percent (%) Rank of <0.5% was considered a strong binding (SB) predictor for MHC class I and a% Rank of <2% was considered an SB predictor for class II. The identified SARS-CoV-2 specific T cell epitopes, their peptide sequences, amino acid positions, location domains, positivity rates in tested patients, and the predicted MHC class I and II alleles are shown in Table 5. This information will be beneficial for further investigation into SARS-CoV-2 -specific T cell responses as well as therapeutic or vaccine development.

3.4. The correlation between SARS-CoV-2-specific T cell response and B cell antibody response

Because most protective B cell antibody responses are dependent upon robust CD4 helper T cell activation and cytokine production, we hypothesized that SARS-CoV-2-specific T cell responses correlate with the virus-specific antibody production in COVID-19-recovered subjects. To test this hypothesis, we measured SARS-CoV-2 humoral immune responses in COVID-19-recovered subjects and examined the relationship between T cell and B cell immune responses. Interestingly, patient P1 showed a marginally positive anti-SARS-CoV-2-RBD IgG titer with 0.229 OD at 450 nm (OD450 cutoff was set at 0.2), whereas patients P7 and P10 were IgG negative (Wang et al., 2021). Notably, all three patients exhibited poor T cell responses to any of the single S peptide stimulations (Fig. 2). These results suggested that SARS-CoV-2 specific antibody production might be dependent upon the virus-specific T cell response in COVID-19 patients. To validate this observation, we



(caption on next page)

Fig. 2. Characterization of SARS-CoV-2 specific T cell response to single S peptides in COVID-19-recovered patients. A-M) Quantification of SFCs from the ELISpot assay of PBMCs derived from four controls (2 HS and 2 Flu patients) and nine COVID-19-recovered patients stimulated with 28 15-mer single S peptides. The dotted line (mean \pm 3SD=20 of all negative control wells) represents the threshold for positive response. The blank/negative control is shown in red. N) Summary data (mean \pm SEM) of SFCs derived from nine COVID-19-recovered patients. O) Representative pseudocolor plots of flow cytometry analysis and gating strategy for lymphocytes, CD4 T cells, CD8 T cells, and expressions of AIM (CD40L and 4-1BB) in CD4 and CD8 T cells. P-Q) Frequencies (%) of CD40L⁺ or CD40L⁺4-1BB⁺ cells within CD4 T cells stimulated by single S peptides detected by flow cytometry. R-S) Frequencies (%) of 4-1BB⁺ or CD40L⁺4-1BB⁺ cells within CD8 T cells stimulated by single S peptides detected by flow cytometry. The dotted line is based on the highest value from the negative controls (blank) and constitutes the positive threshold value in Fig 2. P-S. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

analyzed the relationships between both S and N MP-stimulated T cell responses and anti-S RBD IgG titers as well as the rates of antibody neutralization using Spearman's Correlation analysis. As shown in Fig. 4U-V, both S- and N-specific T cell responses positively correlated with IgG titers from the same subjects. These findings are consistent with previous reports (Grifoni et al., 2020; Ni et al., 2020). Of note, we reported that SARS-CoV-2 neutralizing antibodies positively correlated with the levels of anti-S-RBD IgG antibodies (Wang et al., 2021). We thus further analyzed the correlation between S and N MP-stimulated T cell responses and the antibody neutralization rates and identified positive correlations using this analysis (Fig. 4W-X). These results demonstrate that SARS-CoV-2 humoral immune responses positively correlate with the virus-specific T cell responses in COVID-19-recovered subjects.

4. Discussion

T cells play a pivotal role in fighting SARS-CoV-2 infection and, most likely, in forming immunological memory following recovery from COVID-19. The understanding of T cell immune responses to SARS-CoV-2 and their contribution to protection and disease progression is important for the development of new therapeutics and evaluation of vaccine effectiveness against SARS-CoV-2 infection. In this study, we characterized the memory T cell response to SARS-CoV-2 S and N peptides in COVID-19-recovered patients. Our results demonstrate that virus-specific T cell recall responses are successfully generated in response to SARS-CoV-2 S and N peptide stimulation, but the levels of their overall response are not robust or universal to each viral peptide - as these responses varied amongst different patients. Notably, we have previously shown a positive correlation between the neutralizing antibody titer and anti-Spike S1 IgG concentration (Wang et al., 2021). Also, we found that SARS-CoV-2 specific IgG and IgM antibodies were diminished quickly in some COVID-19-recovered patients, i.e., within weeks to months (Wang et al., 2021). Thus, the longevity/durability of these T cell responses should be investigated further. In essence, our results showing limited virus-specific adaptive immune responses in COVID-19-recovered patients raise concerns about reinfection in COVID-19-recovered patients as well as the efficacy of protection in previously unexposed individuals following the administration of COVID-19 vaccines (To et al., 2020; Brouqui et al., 2021; Kang et al., 2020).

Given that protective antibodies naturally wane over time, the virus-specific T cell memory response is critically valuable to fight against SARS-CoV-2 reinfection and can be used to assess the duration of vaccine protection. Our study revealed that epitope peptides from SARS-CoV-2 S and N proteins can induce virus-specific T cell responses. However, while we found detectable T cell responses to both S and N peptides in COVID-19-recovered patients, none of the single peptides could universally induce T cell responses among all convalescent subjects, indicating that multi-epitope peptides should be used for designing prophylactic vaccines to induce a strong and broad T cell response. It should be pointed out that, without a wild-type viral challenge it is unclear whether these T cell responses - either induced by natural infection or peptide vaccines - can provide sufficient protection or induce pathological effects. In this study, we used both ELISpot and flow cytometry analysis to evaluate the peptide specific T cell response after peptide stimulation. While the ELISpot primarily focuses on IFN- γ secreting cells, the flow analysis emphasizes the markers of CD40L and

4-1BB as peptide-specific T cells. The ELISpot is considered to be more sensitive than flow analysis; however, flow analysis allows for the detection of peptide-specific T cells within CD4 and CD8 populations. Although factors leading to different T cell responses in our COVID-19 patients remain unclear, gender may play a role - given that in this cohort male patients (P2, P6, P8, P10, and P16) appeared to demonstrate a stronger peptide-specific T cell response. Of note, a recent study reported that female patients mount robust T cell responses compared to males in response to SARS-CoV-2 infection (Takahashi et al., 2020), which is opposite of what we observed in the current study. In this context, the limited number of subjects included in our study may have contributed to this outcome. Another important aspect includes the longevity of the protective T-cell responses, which requires further investigation. Some studies reported that a durable T cell response can last for at least six months post-SARS-CoV-2 infection (Tan et al., 2020; Wise, 2020) and that virus-specific T cell responses to SARS-CoV or MERS-CoV can last for 6 or 10 years (Tang et al., 2011; Hilgenfeld and Peiris, 2013). These studies reassure that even if the anti-SARS-CoV-2 IgG antibodies fall below detectable levels within months after infection and possibly vaccination, a robust T cell response can be maintained to combat viral reinfection. Moreover, further studies are needed to determine the molecular mechanisms that support a successful T cell response and to elucidate how pre-existing conditions, co-morbidities, immune status, and other variables can affect protective T cell responses.

Statement of ethics

The study protocol was approved by the Joint Institutional Review Board (IRB) of East Tennessee State University and James H. Quillen VA Medical Center (ETSU/VA; IRB #0519.24 s). Written informed consent was obtained from all participants.

Data availability statement

The datasets generated and analyzed during the course of this study are available from the corresponding author upon reasonable request. The data sharing policies of NIH and VA will be followed.

Credit author statement

Juan Zhao: Conceptualization, Methodology, Formal analysis, Investigation, Data Curation, Writing - Original Draft. Writing - Review & Editing. Ling Wang: Investigation, Writing - Review & Editing. Madison Schank: Investigation, Writing - Review & Editing. Kindi Dang: Investigation, Zeyuan Lu: Investigation. Dechao Cao: Investigation. Sushant Khanal: Investigation. Lam N. Nguyen: Investigation. Lam N.T. Nguyen: Investigation. Jinyu Zhang: Investigation. Yi Zhang: Investigation. James L. Adkins: Resources. Evan M. Baird: Resources. Xiao Y. Wu: Project administration. Shunbin Ning: Writing - Review & Editing, Mohamed El Gazzar: Writing - Review & Editing. Jonathan P. Moorman: Writing - Review & Editing, Funding acquisition. Zhi Q. Yao: Conceptualization, Writing - Review & Editing, Supervision, Funding acquisition

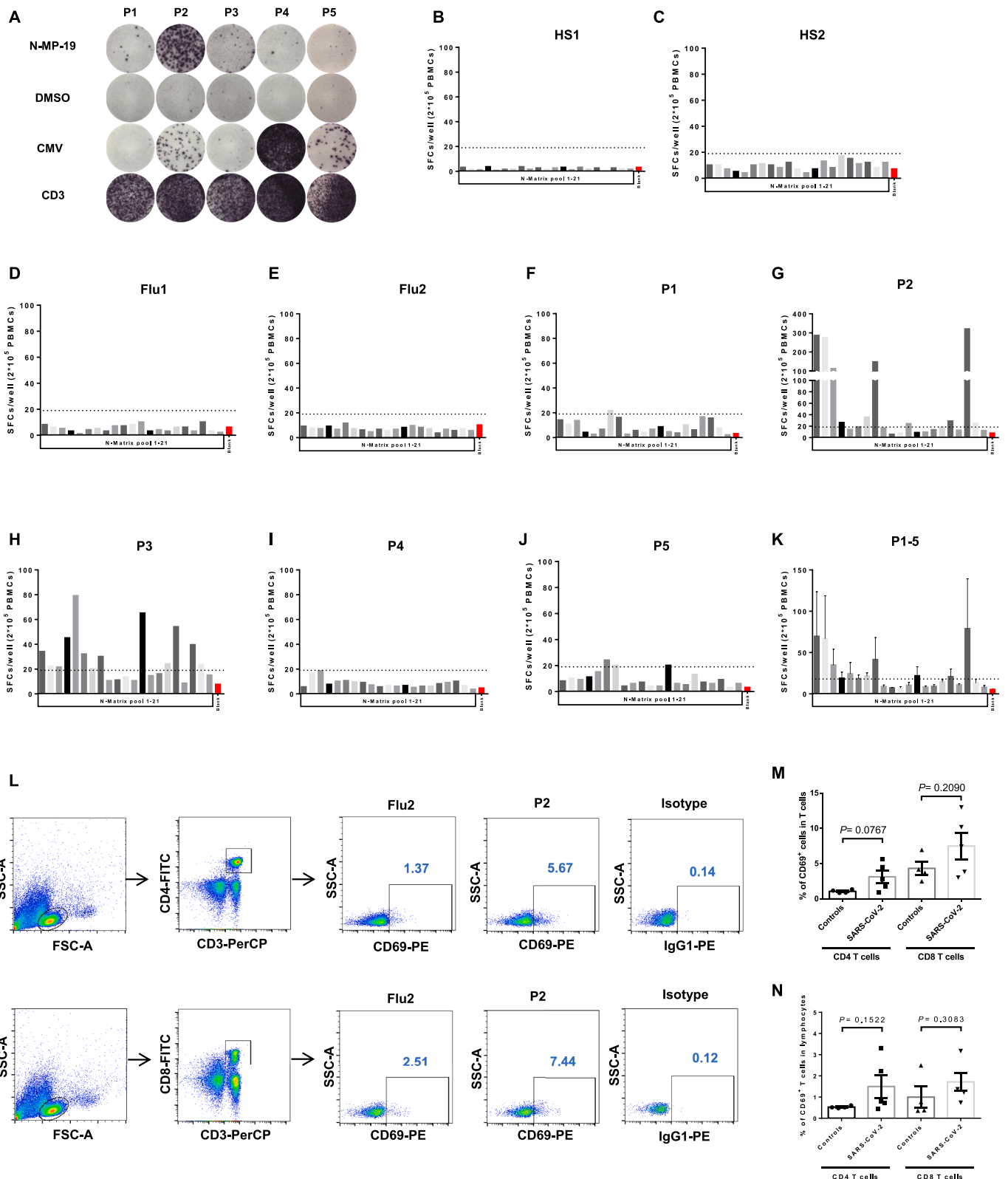
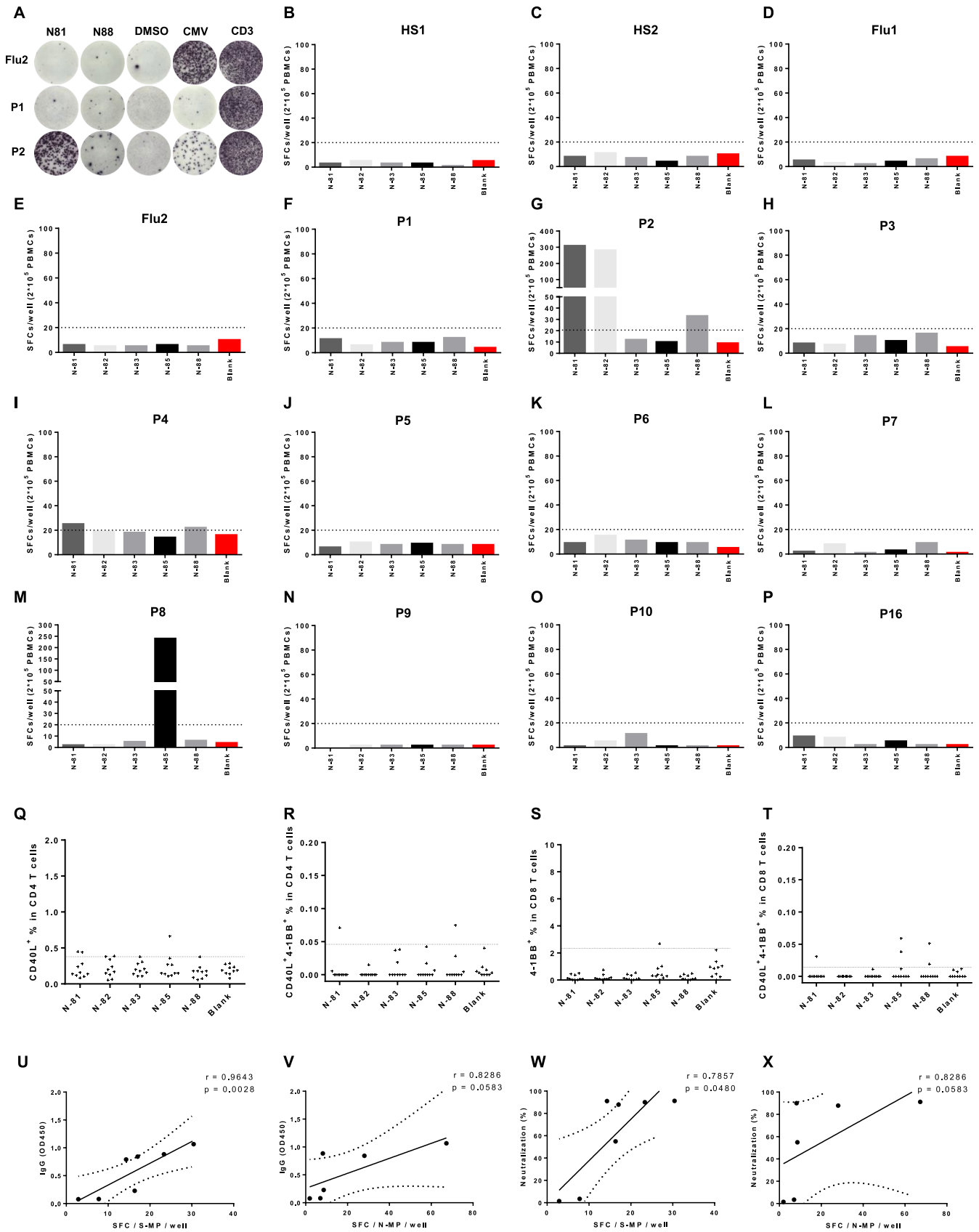


Fig. 3. Detection of SARS-CoV-2 specific T cell response to N peptide pools in COVID-19-recovered patients by IFN- γ ELISpot and flow cytometry. A) Representative results from the ELISpot assay using PBMCs derived from patients P1-P5 that were pulsed with N-MP-19 peptide, DMSO, CMV peptide pools, or CD3-2 mAb. B-J) Quantification of SFCs from the ELISpot assay of PBMCs derived from 4 controls (2 HS and 2 Flu) and five COVID-19-recovered patients. The dotted line (mean + 3SD = 19 of all negative control wells) represents the threshold for positive response. Blank control is shown in red. K) Summary data (mean \pm SEM) of SFCs derived from patients P1-P5. L) Representative pseudocolor plots of flow cytometry analysis and gating strategy for lymphocytes, CD4 T cells, CD8 T cells, and CD69 in T cells in CD4 and CD8 T cells. M) Frequency (%) of CD69⁺ CD4 T cells and CD69⁺ CD8 T cells detected by flow cytometry. N) Frequencies (%) of CD69⁺CD4⁺ T cells and CD69⁺CD8⁺ T cells within lymphocytes detected by flow cytometry. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



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Fig. 4. Identification of SARS-CoV-2 specific T cell response to single N peptide in COVID-19-recovered subjects. A) Representative results from the ELISpot assay of PBMCs from Flu2, P1, and P2 pulsed with N81, N88, DMSO, CMV peptide pools, or CD3–2 mAb. B-P) Quantification of SFCs from the ELISpot assay of PBMCs derived from four controls (2 HS and 2 Flu) and 11 COVID-19-recovered patients pulsed with five single N peptides. The dotted line (mean+3SD=20 of all negative control wells) represents the threshold for a positive response. The blank/negative control is shown in red. Q-R) Frequencies (%) of CD40L⁺ or CD40L⁺4-1BB⁺ cells within CD4 T cells stimulated with five single N peptides detected by flow cytometry. S-T) Frequencies (%) of 4-1BB⁺ or CD40L⁺4-1BB⁺ cells within CD8 T cells stimulated with five single N peptides detected by flow cytometry. The dotted line is based on the highest value from the negative controls (blank) and constitutes the positive threshold value in Fig. 4. F-P. U-V) Spearman’s correlation between IgG antibodies against SARS-CoV-2-S1 RBD in plasma and SFCs induced with S or N matrix pool stimulation. W-X) Spearman’s correlation between the neutralization rates and the SFCs induced with S or N matrix pool stimulation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
HLA allele frequencies in U.S. Caucasian population (n = 61,655).

Allele	% of individuals that have the allele	Allele Frequency (in decimals)	Allele	% of individuals that have the allele	Allele Frequency (in decimals)
A*02:01	47.4	0.2747	DRB1*04:05	0.7	0.0035
A*02:04	0.7	0.0035	DRB1*04:06	0.2	0.001
A*02:05	0.7	0.0035	DRB1*04:07	2.1	0.0105
A*02:06	0.7	0.0035	DRB1*04:08	1.4	0.007
A*25:01	2.2	0.011	DRB1*08:01	4.8	0.0243
A*25:02	0.5	0.0025	DRB1*08:02	0.8	0.004
A*26:01	5.8	0.0294	DRB1*08:03	0.1	0.0005
A*26:05	0.2	0.001	DRB1*08:04	0.3	0.0015
A*26:07	0.2	0.001	DRB1*11:01	10.9	0.056
A*26:08	0.5	0.0025	DRB1*11:02	0.5	0.0025
A*34:01	0.2	0.001	DRB1*11:03	0.7	0.0035
A*34:02	0.2	0.001	DRB1*11:04	4.9	0.0248
A*66:01	0.8	0.004	DRB1*13:01	10.0	0.0513
B*40:01	11.0	0.0566	DRB1*13:02	8.2	0.0418
B*40:02	2.3	0.0115	DRB1*13:03	1.8	0.009
B*45:01	1.2	0.006	DRB1*13:05	0.4	0.002
B*50:02	0.1	0.0005	DRB1*13:20	0.1	0.0005
DRB1*01:01	14.6	0.0758	DRB1*13:29	0.1	0.0005
DRB1*01:02	3.9	0.0196	DRB1*14:01	4.8	0.0243
DRB1*01:03	2.8	0.0141	DRB1*14:02	0.1	0.0005
DRB1*03:01	23.4	0.1247	DRB1*14:04	0.3	0.0015
DRB1*04:01	17.3	0.0906	DRB1*14:07	0.2	0.001
DRB1*04:02	1.9	0.0095	DRB1*15:01	24.8	0.1328
DRB1*04:03	0.5	0.0025	DRB1*16:01	4.7	0.0237
DRB1*04:04	6.1	0.0309			

Table 5
SARS-CoV-2 S and N-specific T cell epitopes.

Peptide Number	Sequence	Sequence range	Located domains	“+” / tested	Potential HLA restriction(s)
S-38	NKSWMESEFRVYSSA	S148-163	S1-NTD	1/9	B*40:02, B*45:01, B*50:02
S-41	SSANNCTFEYVSQPF	S160-175	S1-NTD	1/9	A*26:08, B*40:02
S-42	NCTFEYVSQPFMLDL	S164-179	S1-NTD	4/9	B*40:01, B*40:02
S-43	EYVSQPFMLDLEKQ	S168-183	S1-NTD	1/9	
S-50	IDGYFKIYSKHTPIN	S196-211	S1-NTD	2/9	DRB1_0101, DRB1_1103, DRB1_1601
S-51	FKIYSKHTPINLVRD	S200-215	S1-NTD	2/9	DRB1_0101, DRB1_0103, DRB1_0803
S-80	NFRVQPTESIVRFPN	S316-331	S1-RBD	1/9	A*25:02
S-81	QPTESIVRFPNITNL	S320-335	S1-RBD	2/9	A*25:02, DRB1_0402, DRB1_1501
S-82	SIVRFPNITNLCYFP	S324-339	S1-RBD	2/9	
S-89	WNRKRISNCVADYSV	S352-367	S1-RBD	2/9	
S-90	RISNCVADYSVLYNS	S356-371	S1-RBD	2/9	A*25:01, A*25:02, A*26:01, A*26:05, A*26:07, A*26:08, A*34:01, A*66:01
S-119	YQAGSTPCNGVEGFN	S472-487	S1-RBD	1/9	A*02:06
S-120	STPCNGVEGFNCYFP	S476-491	S1-RBD	1/9	
S-128	RVVLSFELLHAPAT	S508-523	S1-RBD	2/9	B*50:02
S-129	LSFELLHAPATVCGP	S512-527	S1-RBD	2/9	A*02:01, B*50:02, DRB1_0101, DRB1_0102
S-200	FGGFNFSQILPDPK	S796-811	S2	1/9	DRB1_0403, DRB1_0405, DRB1_0406, DRB1_0407, DRB1_0801, DRB1_0803, DRB1_1303, DRB1_1407
S-201	NFSQILPDPKPSKR	S800-815	S2	2/9	DRB1_0301, DRB1_0401, DRB1_0402, DRB1_0404, DRB1_0408, DRB1_1302, DRB1_1329
S-203	PSKPSKRSFIEDLLF	S808-823	S2	1/9	
S-304	PWYIWLGFIAGLIAI	S1212-1227	S2-TM	2/9	
S-307	IAIVMVTIMLCCMSTS	S1224-1239	S2-TM	1/9	
N-81	GMEVTPSGTWLTYTG	N321-335	N-CTD	2/11	A*25:01, A*25:02, A*26:01, A*26:05, A*26:07, A*26:08, A*34:01, A*66:01, B*40:01
N-82	TPSGTWLTYTGAIKL	N324-339	N-CTD	1/11	A*25:01, A*25:02, A*26:01, A*26:05, A*26:08,
N-85	IKLDDKDPNFKDQVI	N337-351	N-CTD	1/11	A*02:01, A*02:06, DRB1_1501
N-88	QVILLNKHIDAYKTF	N348-363	N-CTD	2/11	A26:07, DRB1_1302, DRB1_1329

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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