T-Cell Epitopes in Severe Acute Respiratory Syndrome (SARS) Coronavirus Spike Protein Elicit a Specific T-Cell Immune Response in Patients Who Recover from SARS

Yue-Dan Wang,^{1,2†} Wan-Yee Fion Sin,^{2†} Guo-Bing Xu,^{3†} Huang-Hua Yang,⁴ Tin-yau Wong,⁵
Xue-Wen Pang,¹ Xiao-Yan He,¹ Hua-Gang Zhang,¹ Joice Na Lee Ng,⁴
Chak-Sum Samuel Cheng,² Jing Ju,² Li Meng,² Rui-Feng Yang,³
Sik-To Lai,⁵ Zhi-Hong Guo,⁴ Yong Xie,^{2*}
and Wei-Feng Chen^{1*}

Department of Immunology, Peking University Health Science Centre,¹ and First Hospital, Peking University,³ Beijing, and Department of Biology² and Department of Chemistry,⁴ Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, and Princess Margaret Hospital,⁵ Hong Kong, People's Republic of China

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The immunogenicity of HLA-A2-restricted T-cell epitopes in the S protein of the Severe acute respiratory syndrome coronavirus (SARS-CoV) and of human coronavirus strain 229e (HCoV-229e) was analyzed for the elicitation of a T-cell immune response in donors who had fully recovered from SARS-CoV infection. We employed online database analysis to compare the differences in the amino acid sequences of the homologous T epitopes of HCoV-229e and SARS-CoV. The identified T-cell epitope peptides were synthesized, and their binding affinities for HLA-A2 were validated and compared in the T2 cell system. The immunogenicity of all these peptides was assessed by using T cells obtained from donors who had fully recovered from SARS-CoV infection and from healthy donors with no history of SARS-CoV infection. HLA-A2 typing by indirect immunofluorescent antibody staining showed that 51.6% of SARS-CoV-infected patients were HLA-A2 positive. Online database analysis and the T2 cell binding test disclosed that the number of HLA-A2-restricted immunogenic epitopes of the S protein of SARS-CoV was decreased or even lost in comparison with the homologous sequences of the S protein of HCoV-229e. Among the peptides used in the study, the affinity of peptides from HCoV-229e (H77 and H881) and peptides from SARS-CoV (S978 and S1203) for binding to HLA-A2 was higher than that of other sequences. The gamma interferon (IFN- γ) release Elispot assay revealed that only SARS-CoV-specific peptides S1203 and S978 induced a high frequency of IFN- γ -secreting T-cell response in HLA-A2⁺ donors who had fully recovered from SARS-CoV infection; such a T-cell epitope-specific response was not observed in HLA-A2⁺ healthy donors or in HLA-A2⁺ donors who had been infected with SARS-CoV after full recovery. Thus, T-cell epitopes S1203 and S978 are immunogenic and elicit an overt specific T-cell response in HLA-A2⁺ SARS-CoV-infected patients.

Severe acute respiratory syndrome (SARS) is a severe infectious disease caused by a virus which has been identified through gene sequencing and serological analysis as a new strain of human coronavirus (HCoV) (7, 14, 19). It has been reported that approximately 15 to 30% of common colds are caused by HCoV infections (8, 26). Before the SARS-CoV outbreak, two strains of HCoV had been identified, HCoV-229e and HCoV-OC43 (30). Although *in vivo* and *in vitro* experimental evidence suggested that HCoV is capable of infecting the central nervous system and lung epidermal cells (12, 20, 23), in clinical experience, HCoV usually causes infection only in the upper respiratory tract. However, the newly

emerged SARS coronavirus (SARS-CoV) causes acute severe respiratory symptoms in patients. It was reported that in 25% of SARS-CoV-infected patients the disease progresses to acute lung injury or acute respiratory distress syndrome and results in a mortality rate exceeding 10% (13). Some SARS patients die of complications related to severe acute respiratory distress syndrome, multiorgan failure, disseminated intravascular coagulation, or secondary bacterial septicemic shock.

The coronaviruses are enveloped, positive-stranded RNA viruses associated with various diseases in humans and animals (20, 25, 29). A salient feature is the prominent immune response to the virus after infection. In previous studies of coronavirus, the S protein was identified as a potent immunogen, which induced neutralizing antibodies (3) and elicited cytotoxic T-cell responses (11). As in other HCoV infections, an immune response was generated in SARS-CoV infection patients. The magnitude, specificity, and quality of that immune response are important to pursue, particularly since some infected patients recovered without progressing to the severe phase, whereas some patients had very severe disease. It has been reported that antibodies against coronavirus were de-

* Corresponding author. Mailing address for Wei-Feng Chen: Department of Immunology, Peking University Health Science Centre, 38, Xueyuanlu, Beijing, 100083, People's Republic of China. Phone: (86) 10-82802593. Fax: (86) 10-82802593. E-mail: wfchen@public.bta.net.cn. Mailing address for Yong Xie: Department of Biology, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, People's Republic of China. Phone: (852)2358-7340. Fax: (852)2358-1559. E-mail: Boyxie@ust.hk.

† Y.-D.W., W.-Y.F.S., and G.-B.X. contributed equally to this study.

tected in a patient with SARS. Unfortunately, that study did not show any virus-neutralizing activity of the serum collected during the acute period of SARS (10). In the present study, we focused on anti-SARS-CoV immunity in patients who had recovered from SARS but not in patients suffering acutely from SARS or samples from victims who died of SARS. It is not known whether the patients generated cell-mediated immunity specifically against SARS-CoV which might contribute to the protective immunity.

In order to assess cell-mediated immunity against SARS-CoV antigens, we designed a series of experiments to search for major histocompatibility complex class I-restricted T-cell epitopes with the potential for activating CD8⁺ T cells (2) through a comparison of the structure and immunogenicity of the previously identified coronavirus HCoV-229e with that of the newly isolated SARS-CoV. In the comparison of the amino acid sequence of the S protein in SARS-CoV, which is capable of binding to the HLA-A2 groove, with that in HCoV-229e, a significant difference in the amino acids was found between the two S protein sequences. Using SYFPEITHI and EpiTopePredict (17, 28) to predict HLA-A2 binding affinity, we first obtained the scores for all the T-cell epitopes of both SARS-CoV and HCoV-229e. An alignment between SARS-CoV and HCoV-229e was then performed to search for homologous T epitopes. Homologous T epitopes were found and classified into three types based on the disparate scores between the two viruses.

The three pairs of T-cell epitope peptides were synthesized for validation by biological assays. These assays included a T2 cell binding assay to assess the binding affinity for HLA-A*0201 at the surface of T2 cells and a gamma interferon (IFN- γ) release Elispot assay to assess the CD8⁺ T-cell response to the epitopes. In this study, we tested the immunogenicity of these T-cell epitopes to elicit the specific response of CD8⁺ T cells obtained from blood samples of HLA-A2-positive donors who had recently recovered from SARS and of HLA-A2-positive healthy donors. Using these assays, we were able to reveal two HLA-A2-restricted T-cell epitopes capable of eliciting a CD8⁺ T-cell response in patients who had recovered from SARS-CoV infection.

MATERIALS AND METHODS

Detection of HLA-A2 expression in human PBMCs. Peripheral blood mononuclear cells (PBMCs) isolated from the blood samples of 31 recovered SARS patients, 21 samples from the Peking University First Hospital, Beijing, China, two samples from the Beijing Blood Center, and eight samples from Princess Margaret Hospital, Hong Kong. Collection of PBMC samples was agreed to by patients in writing and authorized by the Hospital Ethics Review Committee. The PBMCs were incubated with anti-HLA-A2 monoclonal antibody (BD Biosciences) at 4°C for 45 min. After washing with 5% newborn calf serum (Sigma)-phosphate-buffered saline, the PBMCs were stained with fluorescein isothiocyanate-conjugated goat anti-mouse antibody (BD Bioscience) at 4°C for 45 min and then washed again with 5% newborn calf serum-phosphate-buffered saline. The fluorescent signals on the cell surface were detected by flow cytometry (FACS-Calibur; Becton Dickinson) and the percent HLA-A2 positive cells was scored.

Prediction of scores of T epitopes on SARS-CoV and HCoV-229e. To identify the nonamer peptides in the amino acid sequences of the spike (S) proteins in SARS-CoV and HCoV-229e through their binding to HLA-A2 molecules, we subjected the virus protein sequences to online database analysis. Binding sequences of the T-cell epitopes were predicted and scored accordingly (6, 27). The homologous sequences of the T-cell epitopes on HCoV-229e and SARS-CoV were found by using Clustal W (1.82) multiple sequence alignments (16). Structural models of the peptides were subsequently constructed and compared with

Hyperchem release 7.0 (1), whereas the structural model of the complex made up of HLA-A2 molecule and a SARS-CoV-specific T-cell epitope, S1203, was constructed with Autodock 2.0 software (9).

Peptide synthesis. Peptides corresponding to the T-cell epitopes and their homologous sequences in both HCoV-229e and SARS-CoV were synthesized and purified to 99.5% (Peptide Synthesis Facility, Department of Chemistry, Hong Kong University of Science and Technology). The lyophilized peptides were dissolved in dimethyl sulfoxide (Sigma) and stored at -70°C at a concentration of 25 mM until used. The Flu matrix peptide p58-66(22) was also synthesized and used as a control peptide in this study.

Detection of T-cell epitope peptide binding to HLA-A2 molecules on T2 cells. T2 cells were cultured in 10% fetal bovine serum-RPMI 1640 medium (Gibco) and incubated with 5% CO₂ at 37°C. Peptides from SARS-CoV and from HCoV-229e were assayed for their ability to bind to and stabilize HLA-A2 molecules on the T2 cells (5). T2 cells (10⁵) suspended in serum-free RPMI 1640 were distributed into 96-well plates (round bottomed; Falcon) and incubated with the peptides (100 mM) supplemented with 5 nM β_2 -microglobulin (Sigma) for 18 h at 37°C. The T2 cells incubated with the peptides were stained with anti-HLA-A2 monoclonal antibody HB-82 (PharMingen), followed by staining with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G antibody (PharMingen). Samples were fixed in 1% paraformaldehyde (Sigma)-phosphate-buffered saline and analyzed by flow cytometry (FACS-Calibur). The number of stabilized HLA-A2 molecules on the surface of T2 cells was determined by fluorescent intensity. The ability of the peptide to combine with the HLA-A2 was calculated as follows: AI = (MFI₁ - MFI₀)/(MFI₂ - MFI₀), where AI is the affinity index for HLA-A2, MFI₁ is the mean fluorescence index (MFI) of staining with HB-82 of T2 cells treated with the peptide, MFI₂ is the MFI of staining with HB-82 of T2 cells without peptide treatment, and MFI₀ is the MFI of T2 cell staining with the isotype immunoglobulin.

IFN- γ -release Elispot assay. The Elispot assay to detect IFN- γ -releasing CD8⁺ T cells was performed by the method described previously (5). Briefly, HLA-A2⁺ PBMCs (10⁵) collected from 12 patients who had been fully recovered for 1 month after SARS infection (Peking University First Hospital) (Table 1) or from eight healthy donors (Hong Kong Red Cross Association and Beijing Blood Center) were plated in triplicate in round-bottomed 96-well plates. The cells were stimulated for 18 h with 50 μ g/ml of the peptides and incubated in 5% CO₂ at 37°C. After the 18-h stimulation, the culture medium was replaced, and the cells were thoroughly mixed and transferred onto flat-bottomed 96-well nitrocellulose plates (HA Multiscreen; Millipore, Bedford, Mass.) coated with anti-IFN- γ monoclonal antibody (2 μ g/ml, 1-D1K; diluted in pH 9.6 carbonate buffer). The cells were then incubated for an additional 20 h. The plates were washed and blocked with 10% human AB serum for 2 h at 37°C. The IFN- γ release Elispots were detected with a commercial kit (Mabtech) according to the instructions of the manufacturer. The averages of the spots in triplicate wells were calculated and expressed as the number of specific IFN- γ -secreting cells/10⁵ cultured PBMC cells (24). The colored spots, representing cytokine-producing cells, were counted with a dissecting microscope. In this study, HLA-A2⁺ PBMCs from two patients who had recovered from SARS and four healthy donors were also assayed; none of the specific spots were detected in these samples (data not shown).

RESULTS

Identification of HLA-A2-positive donors. The 31 samples of HLA-A2⁺ PBMCs obtained from donors who had recovered from SARS infection in Hong Kong and in Beijing were typed for HLA-A2 expression. Sixteen samples were HLA-A2⁺ after staining with anti-HLA-A2 monoclonal antibody and analysis by flow cytometry. The HLA-A2 positivity rate in the donors who had recovered from SARS was 51.6% (not shown), similar to the HLA-A2 positivity rate in the Chinese population (31).

T-cell epitopes predicted by online database analysis. Sequence variation in HLA-A2-associated epitopes was found in SARS-CoV compared with the homologous sequences in HCoV-229e (Fig. 1). The data obtained from the online database analysis displayed three categories of disparate scores of T-cell epitopes between HCoV-229e and SARS-CoV spike protein, as shown in Table 1. According to the online database analysis, more HLA-A2-binding epitopes exist in HCoV-229e

TABLE 1. Patients studied^a

Patient no.	Age (yr)	Gender	Profession	Date of fever initiating	Date of leaving hospital	Date of sample collection	Contact history	Antibodies to SARS-CoV (IgM)	Antibodies to SARS-CoV (IgG)	Clinical symptoms	HLA-A2	SARS-CoV detected by PCR
1	21	F	Nurse(E ¹)	04/23	06/01	06/25	Yes	+	(0.748)	T	+	+
2	21	F	Staff(S ²)	04/21	06/01	06/25	Yes	+	(1.658)	T	+	+
3	21	F	Nurse(S ³)	04/29	06/01	06/25	Yes	+	(1.287)	T	+	+
4	23	F	Nurse(E)	04/23	06/01	06/25	Yes	+	(1.210)	T	+	+
5	28	M	Doctor(S ⁴)	04/23	06/01	06/25	Yes	+	(1.437)	T	+	+
6	28	M	Doctor(S)	05/10	06/01	06/25	Yes	+	(0.726)	T	+	+
7	38	F	Nurse(E)	04/29	06/01	06/25	Yes	+	(1.394)	T	+	+
8	39	F	Nurse(E)	04/23	06/01	06/25	Yes	+	(1.484)	T	+	+
9	40	F	Nurse(E)	04/24	06/01	06/25	Yes	+	(1.624)	T	+	+
10	25	F	Nurse(E)	04/23	06/01	06/25	Yes	+	(1.420)	T	+	+
11	21	F	Nurse(E)	04/23	06/01	06/25	Yes	+	(1.667)	T	+	+
12	23	F	Nurse(E)	03/24	06/01	06/25	Yes ^b	+	(1.680)	T	+	+
C1	20	F	Nurse(E)	04/27	06/01	06/25	Yes	+	(1.575)	S	-	+
C2	52	F	Nurse(O)	04/26	06/01	06/25	Yes ^c	+	(1.457)	S	-	+

^a All patients in our study were medical workers who were exposed to SARS during their work. There were five kinds of positions studied in our research: Nurse(E), nurse who serves in the Department of Emergency; Staff(S), staff member who serves in the SARS Care Unit; Nurse(S), nurse who serves in the SARS Care Unit; Doctor(S), doctor who serves in the SARS Care Unit; and Nurse(O), nurse who serves in some other position. We used the date of fever initiation as the date of SARS initiation. In this study, we used the date of leaving the hospital as the date of recovery. After leaving the hospital all patients had 3 weeks of medical observation, and we collected samples at the end of this period. Immunoglobulin (Ig) M and G antibodies in serum were detected with a kit (Euroimmun Medizinische Labordiagnostika AG); the cutoff was 0.115 (IgM) and 0.135 (IgG) OD₄₅₀ Units, respectively. Clinical symptoms T, typical symptoms of SARS, such as fever and X-ray changes; S, severe symptoms of SARS, such as acute lung injury or acute respiratory distress syndrome; tracheotomy was sometimes necessary for these patients.

^b This nurse, who contacted a SARS patient from Hong Kong, was the first SARS patient among the workers at this hospital.

^c This nurse contacted doctors, who were infected with SARS-CoV from the People's Hospital of Beijing, where there had been a major outbreak of SARS during that time.

S protein than in SARS virus S protein. For example, a 9-amino-acid peptide, H77, in S1 protein of HCoV-229e showed a much higher score than its homologous peptide, S83 (found by Clustal W [1.82] multiple sequence alignments) in SARS-CoV. The amino acid sequence of S83 was entirely different from that of H77. According to the scores, H77 is able to bind to HLA-A2, whereas S83 is not able to bind to the molecule (2). As for amino acid variation in the epitope sequences, one of the epitopes (H881) for binding to HLA-A2 was found on HCoV-229e, and its homologous sequence in the S protein of SARS-CoV (S978) was also found to be capable of binding to HLA-A2. Although there were two amino acid differences between these peptides, both epitopes bound to HLA-A2 with similarly high binding affinity (3).

The occurrence of a new epitope, S1203, in SARS-CoV was first revealed by our online database analysis. Its homologous sequence, H1121, on HCoV-229e did not bind to HLA-A2 molecules. In this nonamer peptide, there were six amino acid differences. The structure of these peptides was analyzed with Hyperchem release 7.0 software. Figure 1 shows that the HLA-A2-associated epitopes S978 and S1203 both have leucine or isoleucine in position 2 and valine or leucine in position 9 (Fig. 2A and B); this is compelling evidence for major histocompatibility complex binding, as pointed out in previous references (4, 5, 6, 27). Similar structures were found in H77 and H881 but not in the homologous sequences of H77 and S1203 (S83 and H1121, respectively). According to docking analysis, SARS-CoV-specific epitopes S978 and S1203 were able to form a stable complex with the HLA-A2 molecule when it bound to the peptide combination site on HLA-A2 (Fig. 2C and D).

T2 cell binding assay. The T2 cell binding assay was employed to validate the binding affinity of the peptides to HLA-A2 molecules predicted by database analysis. The binding affinity of peptide H77 of the S protein of HCoV-229e was higher than that of the homologous sequences of S83, whereas the binding affinity of H881 of HCoV-229e was slightly higher than that of S978 of the S protein of SARS-CoV. In sharp contrast, the binding affinity for HLA-A2 molecules of peptide S1203 of the S protein of SARS-CoV was considerably higher than that of its homologous sequence H1121 of the S protein of HCoV-229e, with H1121 being virtually unable to bind to HLA-A2 molecules (Table 2).

T-cell-specific immune response to the HLA-A2 binding peptides of S protein of SARS-CoV. The HLA-A2-restricted T-cell epitope T-cell immune response was assessed by an IFN- γ release Elispot assay. Employed as a positive control in parallel assays, the flu matrix peptide p58-66 (GILGFVFTL) induced a similarly moderate level of IFN- γ release-specific T-cell response in PBMC samples obtained both from donors who had recovered from SARS and from healthy donors in the Elispot assay. In the experimental assays, SARS-CoV S protein containing the specific peptide S1203 elicited a high IFN- γ release-specific T-cell response in PBMCs obtained from donors who had fully recovered from SARS. All 12 HLA-A2⁺ donors who had recovered from SARS-CoV infection mounted a T-cell response to S1203, with an average number of spot-forming cells of $65 \pm 48/10^5$ cultured PBMC cells, ranging from 7 to 149/10⁵ cultured PBMC cells (Fig. 3). Such

HCoV-229e	MFVLLVAYALLH---IAGCQTNGNLNTS----YSVCNGCVGYSENVFAVESGGYIPSDFA	53
SARS	MFIFLLFLLTSLGSDLDRCCTTFDDVQAPNYTQHTSSMRGVVYPDEIFRSDT-LYLTQDLF	59
	**::*: :* : * * . : : . : : * * . : : * : : *	
HCoV-229e	FNNWFLNTNTSSVVDGVVRSFQPLLLNCLWSVSG-LRFTTGfVYfNGTGRGCKGFSSDV	112
SARS	LPFYSNVTGFHTINHTFGNPVIFPKDGIYFAATEKSNVVRGWVFGSTMNKSQSVIIINN	119
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HCoV-229e		842
SARS		939
HCoV-229e	SLNHLTSQLRQNFQAISSSIQAIDRLDTIQADQQVDRLITGRLAALNVFVSHTLTKEYE	902
SARS	ALNTLVKQLSSNFGAISSVLNDILSRDKVEAEVQIDRLITGRQLSLQTYVTQQLIRAAE	999
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HCoV-229e		1082
SARS		1162
HCoV-229e	YTVQKLQTLIDNINSTLVDLKWLNRVETIKWPWWVLCISVVLIFVVSMLLLCCSTGC	1142
SARS	KEIDRLNEVAKNLNESLIDLQELGKYEQYIKWPWVWLGFIAGLIAIVMTILCCMTSC	1222
	: : * : : * : * : * : * : * * : : * : * : * : * : *	
HCoV-229e	CGFFS--CFASSIRGCCSTKLPYYDVEKIHIQ	1173
SARS	CCLKGACSCGSCCKFDEDDSEPVLKGVKLHYT	1255
	* . : . * . * * . * . * : *	

FIG. 1. Alignment of S protein of HCoV-229e and SARS-CoV analyzed by Clustal W (1.82). *, same amino acid residue. :, related amino acid residue. The underlined sequences are the epitopes and their homologous sequences on HCoV-229e.

a T-cell response was not detected in the PBMCs obtained from healthy donors.

The S978 T-cell epitope in the S protein of SARS-CoV elicited a relatively high T-cell response by IFN- γ release, with an average number of spot-forming cells of $47 \pm 39/10^5$ cultured PBMC cells, ranging from 6 to $113/10^5$ cultured PBMC cells (Fig. 3). Interestingly, S1203 and S978 elicited the highest T-cell response in PBMCs obtained from six HLA-A2⁺ donors, who had recovered from SARS within 1 month of the test, with average number of spot-forming cells being $100 \pm 25/10^5$ and $73 \pm 26/10^5$ cultured PBMC cells, respectively, ranging from 80 to $149/10^5$ and 41 to $113/10^5$ cultured PBMC cells, respectively. In contrast, its homologous sequence of T-cell epitope, H881 of HCoV-229e, induced a very weak IFN- γ release T-cell response in PBMCs from donors who had fully recovered from SARS; such a weak T-cell response was also shown in the PBMCs of healthy donors.

The T-cell epitope H77 of HCoV-229e induced a low IFN- γ release T-cell response in PBMCs from donors who had fully recovered from SARS and a weak T-cell response in PBMCs from healthy donors. Peptides corresponding to the epitopes without HLA-A2 binding ability, such as S83 and H1121, were unable to elicit a specific IFN- γ release T-cell response at all in PBMC samples obtained from donors who had fully recovered

from SARS or from healthy donors, with the spot number at the basal level, i.e., the number of spots in the group without peptide stimulation (Fig. 3). Moreover, the Elispot assay was also done with PBMCs obtained from two HLA-A2⁺ donors who had fully recovered from SARS. There was no positive response to the S1203 and S978 peptides (data not shown).

DISCUSSION

SARS-CoV caused an epidemic between March and June 2003, with 8,450 people infected worldwide. The majority of patients experienced high fever and severe pneumonia. Two other types of coronavirus infection are known to cause 15 to 30% of all common colds in humans. Neutralizing antibody is one of the major protective factors against virus infection; the cytotoxic T-lymphocyte response is the other major defense specific against viral infection. The synergetic effects of both specific neutralizing antibody and the cytotoxic T-lymphocyte response exert potent immunity against the virus infection. The SARS-CoV is a new type of coronavirus (19). Coronavirus infection in humans may induce immunological memory. However, the outbreak of SARS in early 2003 indicates that immunity against previous coronavirus infections offered no protective immunity against SARS-CoV infection. It was noticed that

FIG. 2. Structural model constructed by computer analysis. The S978 (A) and S1203 (B) epitopes were used in this study. They had a similar structure, with leucine or isoleucine in position 2 and valine or leucine in position 9 as well as in the positive control, the influenza virus peptide 58-66. (C and D) Complex formed by HLA-A2- and SARS-specific epitopes S978 and S1203, which is shown in yellow. S978 and S1203 adapt to combine into the antigen peptide binding site of HLA-A2.

no significant major genomic rearrangements or large insertions or deletions in the genes coding for the replicase, S, E, M, or N proteins were found (19), but analysis of HLA-A2-restricted T-cell epitopes of the S protein of HCoV229e and SARS-CoV by alignment of the amino acid sequences of these two virus strains based on the online database unveiled great changes in T-cell epitopes. A dramatic feature is that many

T-cell epitopes in the S protein of HCoV229e are no longer present in the S protein of SARS-CoV. Although there has been no report indicating that a T-cell immune response is induced by coronavirus peptides, the S protein of coronavirus was observed to elicit the cytotoxic T-lymphocyte response in vivo (11). With the intention of demonstrating a CD8⁺ T-cell response in SARS-CoV-infected

TABLE 2. Affinity of synthesized epitopes for HLA-A2 predicted by online database analysis and tested with the T2 cell binding assay

Epitope	Sequence	SYFPEITHI score	EpitopePredict score	T2 binding result assay (affinity index)
H77	LLNCLWSV (HCoV-229e, 77–85)	30	151	7.51
S83	FKDGIYFAA (SARS-CoV, 83–91)	8	63	1.72
H881	LITGRLAAL (HCoV-229e, 881–889)	28	104	9.92
S978	LITGRLQSL (SARS-CoV, 978–986)	27	99	10.46
H1121	ISVVLIFVV (HCoV-229e, 1121–1129)	17	92	2.15
S1203	FIAGLIAIV (SARS-CoV, 1203–1211)	30	121	9.18
58-66	GILGFVFTL (influenza virus, 58–66)	30	107	8.12

FIG. 3. Elispot assay. (A) Spots in the Elispot assay with PBMCs obtained from HLA-A2⁺ donors who had fully recovered from SARS for 1 month. The peptides used in the Elispot assay are marked. PBMCs from SARS patients could be activated by epitopes from SARS virus (S978 and S1203), and PBMCs could be activated slightly by the influenza virus peptide epitope 58-66. PBMCs did not secrete IFN- γ without the peptide or when the sequences homologous to S978 and S1203 from HCoV-229e were added to the culture system. (B) The spots are a measure of IFN- γ secretion from PBMCs. The black bar shows the PBMCs from 12 patients who had recovered from SARS, and the open bars show the PBMCs from eight healthy HLA-A2⁺ donors. SARS-CoV-specific peptides S1203 and S978 induced much greater IFN- γ secretion in the PBMCs from donors who had recovered from SARS than in those from healthy donors. Two peptides from HCoV-229e induced moderate IFN- γ -secreting cells in the PBMC from patients who had recovered from SARS infection. Their homologous sequences, non-HLA-A2 associated peptides, induced lower IFN- γ secreting cells or did not induce any IFN- γ -secreting cells in PBMCs from patients who had recovered from SARS, healthy donors,

patients, we took three steps: sequence alignment and prediction of HLA-A2-restricted T-cell epitopes; T2 cell binding assay to validate binding affinity; and functional assay by IFN- γ release Elispot assay to estimate the CD8⁺ T effector cell-specific responsive to the peptides. In the Elispot assay that we employed, only the memory T cells or the survival effector T cells responsive to the peptide can be detected (24). The IFN- γ release Elispot assay was validated the HLA-A2-restricted influenza virus matrix protein p58-66 as a positive control (18).

By database analysis and as validated by the T2 binding assay, two HLA-A2-restricted T-cell epitopes, S1203 and S978, were found in the S protein of SARS-CoV. Both T-cell epitopes are immunogenic, capable of provoking a potent T-cell response. As these peptides are presented only by HLA-A2 molecules, the responsive T cells must belong to the CD8⁺ T-cell subset. S1203 is a newly generated T-cell epitope of a 9-amino-acid peptide, which has no amino acid identical to its homologous sequence in the S protein of HCoV-229e. S978 is also a nanomer with identical amino acids at positions 2 and 9 anchoring in the HLA-A2 peptide binding groove. There are only two amino acid differences between S978 and H881. These distinct amino acid residues probably constitute the T-cell receptor binding motif and are crucial for the immunogenicity of the S978 peptide stimulating a T-cell response in SARS patients, whereas its homologous peptide, though having a high binding affinity for HLA-A2 molecules, induced only a weak response in SARS patients. Like the weak response induced by H881, a low to weak response to H77, a T-cell epitope of the S protein of HCoV-229e, was also detected in SARS patients. However, such a weak to low T-cell response was also detected in healthy donors and may represent an anamnestic reaction to previous infection with HCoV-229e or a cross-reaction between HCoV-229e and SARS-CoV (15). By contrast, the H881-homologous sequence S978 is a potent immunogenic T-cell epitope of the S protein of SARS-CoV revealed by our assays.

Both the S978 and S1203 T-cell epitopes are localized in the S2 protein of SARS-CoV. The function of the S2 protein is to fuse with the cognate receptor on the membrane of host cells to allow the virus to enter the cells. The ability of S978 and S1203 to elicit the CD8⁺ T-cell response may induce protective immunity and lead to killing of the invading virus. These two T-cell epitope peptides are obviously good candidates for at least the partial constitution of an anti-SARS-CoV vaccine. Our identification of T-cell epitopes of the S protein of SARS-CoV also provides an important tool for monitoring the duration of postinfection immunity in parallel with the measurement of neutralizing antibody.

The dramatic changes in the T-cell epitopes of SARS-CoV in comparison with those of HCoV-229e and HCoV-OC43 may be a major cause of the SARS outbreak. Such changes may play a role in viral virulence. There was no or only a weak anamnestic immune response to the T-cell epitopes of the S protein of HCoV-229e, indicating short-lasting immunity after

or the nonpeptide group. As a positive control peptide, influenza virus peptide 58-66 induced moderate levels of IFN- γ -secreting cells in HLA-A2-positive PBMCs both from patients who had recovered from SARS and from healthy donors.

infection with the other coronavirus. Unlike previous coronavirus infections, SARS-CoV infection apparently induced strong and long-lasting immunity. It is at present not clear how long this immunity will last, and donors who have fully recovered from SARS will require further monitoring.

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REFERENCES

- Bartulewicz, D., A. Markowska, S. Wolczynski, M. Dabrowska, and A. Rozanski. 2000. Molecular modelling, synthesis and antitumour activity of carbocyclic analogues of netropsin and distamycin—new carriers of alkylating elements. *Acta Biochim. Pol.* **47**:23–35.
- Cerundolo, V., J. Alexander, K. Anderson, C. Lamb, P. Cresswell, A. Michael, F. Gotch, and A. Townsend. 1990. Presentation of viral antigen controlled by a gene in the major histocompatibility complex. *Nature (London)* **345**:449–452.
- Collins, A. R., R. L. Knobler, H. Powell, and M. J. Buchmeier. 1982. Monoclonal antibodies to murine hepatitis virus-4 (strain JHM) define the viral glycoprotein responsible for attachment and cell fusion. *Virology* **119**:358–371.
- Gnjatic, S., D. Atanackovic, M. Matsuo, E. Jager, S. Y. Lee, D. Valmori, Y. T. Chen, G., Ritter, A. Knuth, and L. J. Old. 2003. Cross-presentation of HLA class I epitopes from exogenous N. Y.-ESO-1 polypeptides by nonprofessional APCs. *J. Immunol.* **170**:1191–1196.
- Gricks, C. S., E. Rawlings, L. Foroni, J. A. Madrigal, and P. L. Amlot. 2001. Somatic mutated regions of immunoglobulin on human b-cell lymphomas code for peptides that bind to autologous major histocompatibility complex class I, providing a potential target for cytotoxic T cells. *Cancer Res.* **61**:5145–5152.
- Hansson, L., H. Rabbani, J. Fagerberg, A. Osterborg, and H. Mellstedt. 2003. T-cell epitopes within the complementarity-determining and framework regions of the tumor-derived immunoglobulin heavy chain in multiple myeloma. *Blood* **101**:4930–4936.
- Holmes, K. V. 2003. SARS-associated coronavirus. *N. Engl. J. Med.* **348**:1948–1951.
- Holmes, K. V., and M. M. Lai. 1996. *Coronaviridae*: the viruses and their replication, p. 1075–1094. In B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), *Fields virology*, 3rd ed. Lippincott-Raven, Philadelphia, Pa.
- Jois, S. D., and T. J. Teruna. 2003. A peptide derived from LFA-1 protein that modulates T-cell adhesion binds to soluble ICAM-1 protein. *J. Biomol. Struct. Dyn.* **20**:635–644.
- Ksiazek, T. G., D. Erdman, C. S. Goldsmith, et al. 2003. A novel coronavirus associated with severe acute respiratory syndrome. *N. Engl. J. Med.* **348**:1953–1966.
- Kyuwa, S., and S. A. Stohman. 1990. Pathogenesis of a neurotropic murine coronavirus strain, JHM, in the central nervous system of mice. *Semin. Virol.* **1**:273–280.
- Lachance, C., N. Arbour, N. R. Cashman, and P. J. Talbot. 1998. Involvement of aminopeptidase N (CD13) in infection of human neural cells by human coronavirus 229E. *J. Virol.* **72**:6511–6519.
- Lew, T. W., T. K. Kwek, D. Tai, et al. 2003. Acute respiratory distress syndrome in critically ill patients with severe acute respiratory syndrome. *JAMA* **290**:374–380.
- Marra, M. A., S. J. Jones, C. R. Astell, et al. 2003. The genome sequence of the SARS-associated coronavirus. *Science* **300**:1399–1404.
- Nilges, K., H. Hohn, H. Pilch, C. Neukirch, K. Freitag, P. J. Talbot, and M. J. Maeurer. 2003. Human papillomavirus type 16 E7 peptide-directed CD8⁺ T cells from patients with cervical cancer are cross-reactive with the coronavirus NS2 protein. *J. Virol.* **77**:5464–5474.
- Ramirez, E., Cartier, L., Villota, C., Fernandez, J. 2003. Genetic characterization and phylogeny of human T-cell lymphotropic virus type I from Chile. *Virus Res.* **84**:135–149.
- Rammensee, H., J. Bachmann, N. P. Emmerich, O. A. Bachor, and S. Stevanovic. 1999. SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics* **50**:213–219.
- Rivoltini, L., D. J. Loftus, K. Barracchini, et al. 1996. Binding and presentation of peptides derived from melanoma antigens MART-1 and glycoprotein-100 by HLA-A2 subtypes. Implications for peptide-based immunotherapy. *J. Immunol.* **156**:3882–3891.
- Rota, P. A., M. S. Oberste, S. S. Monroe, et al. 2003. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. *Science* **300**:1394–1399.
- Siddell, S. G., H. Wege, and V. ter Meulen. 1983. The biology of coronaviruses. *J. Gen. Virol.* **64**:761–776.
- Stewart, J. N., S. Mounir, and P. J. Talbot. 1992. Human coronavirus gene expression in the brains of multiple sclerosis patients. *Virology* **191**:502–505.
- Storkus, W. J., H. J. Zeh III, R. D. Salter, and M. T. Lotze. 1993. Identification of T-cell epitopes: rapid isolation of class I-presented peptides from viable cells by mild acid elution. *J. Immunother.* **14**:94–103.
- Talbot, P. J., S. Ekande, N. R. Cashman, S. Mounir, and J. N. Stewart. 1993. Neurotropism of human coronavirus 229E. *Adv. Exp. Med. Biol.* **342**:339–346.
- Tanaka, Y., S. Dowdy, D. C. Linehan, T. J. Eberlein, and P. S. Goedegebuure. 2003. Induction of antigen-specific CTL by recombinant HIV transactivating fusion protein-pulsed human monocyte-derived dendritic cells. *J. Immunol.* **170**:1291–1298.
- Tyrell, D. A. J., J. D. Almeida, D. M. Berry, C. H. Cunningham, D. Hamre, M. S. Hofstad, L. Malluci, and K. McIntosh. 1968. Coronaviruses. *Nature (London)* **220**:650.
- Vabret, A., T. Mourez, S. Gouarin, J. Petitjean, and F. Freymuth. 2003. An outbreak of coronavirus OC43 respiratory infection in Normandy, France. *Clin. Infect. Dis.* **36**:985–989.
- Wang, Q. J., X. L. Huang, G. Rappocciolo, et al. 2002. Identification of an HLA A*0201-restricted CD8⁺ T-cell epitope for the glycoprotein B homolog of human herpesvirus 8. *Blood* **99**:3360–3366.
- Wang, Y., Y. Xie, and W. Chen. 2003. Immunoinformatic analysis for the epitopes on SARS virus surface protein. *Beijing Da Xue Xue Bao* **35**(Suppl.):70–71.
- Wege, H., S. G. Siddell, and V. ter Meulen. 1982. The biology and pathogenesis of coronaviruses. *Curr. Top. Microbiol. Immunol.* **99**:165–200.
- Wentworth, D. E., and K. V. Holmes. 2001. Molecular determinants of species specificity in the coronavirus receptor aminopeptidase N (CD13): influence of N-linked glycosylation. *J. Virol.* **75**:9741–9752.
- Zhang, H. G., X. W. Pang, X. Y. Shang, Q. Xing, and W. F. Chen. 2003. Functional supertype of HLA-A2 in the presentation of Flu matrix p58–66 to induce CD8⁺ T-cell response in a Northern Chinese population. *Tissue Antigens* **62**:285–295.