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Screening and Identification of Severe Acute Respiratory Syndrome-Associated Coronavirus-Specific CTL Epitopes¹

Minghai Zhou,^{*†} Dongping Xu,[†] Xiaojuan Li,^{*†} Hongtao Li,^{*†} Ming Shan,^{*} Jiaren Tang,[‡] Min Wang,[†] Fu-Sheng Wang,[†] Xiaodong Zhu,^{*†} Hua Tao,^{*} Wei He,[‡] Po Tien,^{2*} and George F. Gao^{2*§}

Severe acute respiratory syndrome (SARS) is a highly contagious and life-threatening disease that emerged in China in November 2002. A novel SARS-associated coronavirus was identified as its principal etiologic agent; however, the immunopathogenesis of SARS and the role of special CTLs in virus clearance are still largely uncharacterized. In this study, potential HLA-A*0201-restricted spike (S) and nucleocapsid protein-derived peptides were selected from an online database and screened for potential CTL epitopes by *in vitro* refolding and T2 cell-stabilization assays. The antigenicity of nine peptides which could refold with HLA-A*0201 molecules was assessed with an IFN- γ ELISPOT assay to determine the capacity to stimulate CTLs from PBMCs of HLA-A2⁺ SARS-recovered donors. A novel HLA-A*0201-restricted decameric epitope P15 (S411–420, KLPDDFMGCV) derived from the S protein was identified and found to localize within the angiotensin-converting enzyme 2 receptor-binding region of the S1 domain. P15 could significantly enhance the expression of HLA-A*0201 molecules on the T2 cell surface, stimulate IFN- γ -producing CTLs from the PBMCs of former SARS patients, and induce specific CTLs from P15-immunized HLA-A2.1 transgenic mice *in vivo*. Furthermore, significant P15-specific CTLs were induced from HLA-A2.1-transgenic mice immunized by a DNA vaccine encoding the S protein; suggesting that P15 was a naturally processed epitope. Thus, P15 may be a novel SARS-associated coronavirus-specific CTL epitope and a potential target for characterization of virus control mechanisms and evaluation of candidate SARS vaccines. *The Journal of Immunology*, 2006, 177: 2138–2145.

An unusual atypical pneumonia or severe acute respiratory syndrome (SARS)³ emerged in China in November 2002 and spread to 30 countries in early 2003 (1–5). This disease is characterized by progressive respiratory failure and death in ~10% of cases and has garnered significant attention from many public health, social, economic, and political institutions worldwide due to its feared pandemic potential. Through the efforts of an international consortium of laboratories, the causative agent of SARS was identified as a new type of coronavirus, SARS-

associated coronavirus (SARS-CoV) (6–10). However, the emergence of SARS-CoV is not well-understood and its apparent presence in animal reservoirs provides a continued threat of re-emergence, possibly in forms with increased infectivity (11, 12).

The immunopathogenesis of SARS is still poorly characterized. It has been reported that some patients experience deterioration during the second week of illness despite a decreasing viral load and it has been suggested that there may be an immunopathologic component contributing to lung damage (13–15).

Lymphocytopenia is common among SARS patients, with a notable drop in CD4⁺ and CD8⁺ T cell counts occurring early in the course of the disease and contributing to disease pathology (3, 10, 13, 16–18). Specific HLA class I alleles have been reported to correlate with SARS susceptibility (19, 20). Recent studies have demonstrated that high-avidity CTLs are essential for the effective clearance of viral infections. Therefore, as with many other viral infections, T cell immunity is likely to be a critical factor for protection from SARS-CoV infection as well. However, due to the lack of identified SARS-CoV-specific CTL epitopes, the role of CTLs in immunopathogenesis and their function in viral clearance remain unclear. In addition, with the recent development of several vaccines against SARS-CoV, there is currently a need for a reliable markers to indicate effective SARS-CoV T cell responses (21–23). Hence, the identification of specific CTL epitopes should be of great use for both understanding viral control mechanisms and SARS vaccine evaluation.

The antigenicity of SARS-CoV seems to be largely dependent upon two viral proteins that comprise the nucleocapsid and the envelope spike. The nucleocapsid (N) protein has been demonstrated to be highly immunogenic and seems to be an important component of the humoral response to SARS-CoV (24, 25). The envelope spike (S) protein is a large type-I transmembrane glycoprotein which is not only responsible for receptor binding and

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³ Abbreviations used in this paper: SARS, severe acute respiratory syndrome; SARS-CoV, SARS-associated coronavirus; S, spike; N, nucleocapsid; HBcAg, hepatitis virus B core Ag; β_2 m, β_2 -microglobulin; SFC, spot-forming cell; Tg, transgenic; FI, fluorescence index.

membrane fusion, but also serves as a potent immunogen that induces neutralizing Abs (24, 26). This protein contains two functional domains, S1 and S2, which are located in the N- and C-terminal regions, respectively. It has been previously demonstrated that the angiotensin-converting enzyme 2 on the cell surface functions as a receptor for the SARS-CoV S protein (27, 28). A 193-aa small fragment within the S1 domain (S318–510) was identified as a minimal receptor domain and contained multiple conformation-dependent epitopes that induce highly potent neutralizing Abs (29, 30). Recently, three S protein-derived CTL epitopes SSp-1 (S1167), S987, and S1203 were identified by two groups (31, 32). These epitopes, however, were all screened from nonamer libraries. Because CTL epitopes are also known to occur as decamers (33–37), the possible existence of decameric epitopes in SARS-CoV remains to be explored. In addition, all three identified epitopes were localized in the S2 domain. Whether the receptor-binding S1 domain and immunodominant N protein contain CTL epitopes is currently unknown.

Here, we studied a panel of SARS-CoV S and N protein-derived peptides to identify those with binding motifs for the HLA-A*0201 molecules. HLA-A2 is the most common HLA-A allele in Asian populations, particularly in the Chinese, with an estimated frequency of ~50%. It is also the most extensively studied HLA class I molecule (38). We first applied bioinformatics to predict possible CTL epitopes and followed by refolding these predicted peptides with HLA-A*0201 and β_2m microglobulin (β_2m) molecules. Next, we evaluated the binding capacity of all refoldable peptides with HLA-A*0201 molecules on the T2 cell surface. These peptides were also tested for the ability to stimulate IFN- γ -producing CTLs of PBMCs isolated from HLA-A2⁺ donors previously infected with SARS-CoV. Using this assay, we found that peptide P15 (S411–420, KLPDDFMGCV) was able to elicit an effective and specific CTL reaction. The existence of P15-specific CTL was also confirmed by tetramer staining. Finally, we evaluated the ability of peptide P15 to provoke in vivo-specific CTL responses in HLA-A2.1/K^b-transgenic (Tg) mice immunized with the P15 peptide or the DNA vaccine encoding the S protein of SARS-CoV. Substantial P15 epitope-specific CTLs were generated by the mice as detected by IFN- γ release ELISPOT assay, intracellular IFN- γ , and tetramer staining.

Materials and Methods

Prediction of epitopes and synthesis of peptides

Sequences of potential HLA-A*0201-binding peptides within SARS-CoV S and N proteins were based on the BJ01 strain of SARS-CoV (GenBank accession no. AY278488). A computer-based program was applied with access through the website of Bioinformatics and Molecular Analysis Section (BIMAS) HLA Peptide Binding Predictions (39). Nine- and 10-mer peptides with high estimated half-time of dissociation ($T_{1/2}$) were synthesized and the purity was determined to ~90% by analytical HPLC profile and mass spectrometry. As a positive control for HLA-A*0201-binding ability, the HLA-A*0201-restricted hepatitis B virus core Ag (HBcAg)-derived peptide HBVC-1 (FLPSDFPFSV) was used (34). HBcAg-derived H-2K^d-restricted peptide HBVC-2 (SYVNTNMGL) (40) was used as a negative control. Lyophilized peptides were stored in aliquots at -80°C .

Detection of HLA-A2 expression in human PBMCs

PBMCs were isolated from the blood samples of 10 SARS-recovered donors from the Beijing 302 Hospital (Beijing, China). Consent for the collection of PBMC samples was given by each patient in writing and authorized by the Hospital Ethics Review Committee. The PBMCs were incubated with FITC-labeled anti-HLA-A2 mAb BB7.2 (BD Pharmingen) at 4°C for 45 min. Fluorescent signals on the cell surface were detected using a FACSCalibur flow cytometer (BD Biosciences) and data were analyzed using CellQuest software (BD Biosciences).

Refolding of computer-predicted peptide with HLA-A*0201 H chain and β_2m

Refolding was performed as previously described with minor modifications (41, 42). Briefly, HLA-A*0201 H chain and β_2m were expressed in *Escherichia coli* with the pET prokaryotic expression system (R&D Systems) and isolated from the inclusion bodies. The inclusion bodies of HLA-A*0201 H chain and β_2m were separately dissolved in a solution of 10 mM Tris-HCl (pH 8.0) and 8 M urea. The synthetically prepared peptide was dissolved in DMSO. HLA-A*0201 H chain, β_2m , and peptide were mixed at a molecular ratio of 1:1:3 in a diluted solution of 200-ml volume. After placing at 4°C for 24–48 h, the soluble portion was concentrated and then purified by chromatography on a Superdex G-75 size exclusion column (Amersham Pharmacia Biotech).

Detection of the binding of potential T cell epitopic peptides to HLA-A*0201 molecules on T2 cells

The T2 cell line was a gift from Prof. W. Chen (Peking University Health Science Center, Beijing, China). A peptide-induced stabilization assay of the HLA-A*0201 molecules expressed by T2 cells was performed using a previously described method (43–45). Briefly, T2 cells were incubated with 50 μM candidate peptides and 1 μM human β_2m (Sigma-Aldrich) in serum-free RPMI 1640 medium (Invitrogen Life Technologies) for 18 h at 37°C in a 5% CO_2 incubator. Expression of HLA-A*0201 on T2 cells was then determined by staining with FITC-labeled anti-HLA-A2 mAb BB7.2 and detected by flow cytometry using FACScan (BD Biosciences). Data analysis was performed using CellQuest software. The fluorescence index (FI) was calculated as follows: FI = (mean FITC fluorescence with the given peptide – mean FITC fluorescence without peptide)/(mean FITC fluorescence without peptide). Peptides with FI ≥ 1 were regarded as high-affinity candidate epitopes.

In vitro stimulation of IFN- γ -producing CTLs with selected peptides

PBMCs were separated from the whole blood of four HLA-A2⁺ and six HLA-A2⁻ SARS-recovered donors as well as four HLA-A2⁺ healthy controls. The SARS-recovered donors were sampled 7–8 mo after their onset of disease. PBMCs ($2 \times 10^6/\text{ml}$) were cultured with each of the HLA-A*0201 refolding peptides at a concentration of 10 μM in RPMI 1640 medium containing 10% FCS and 20 U/ml recombinant human IL-2 (rhIL-2) in 24-well culture plate. Half of the medium was changed at day 4 with supplementation of rhIL-2 at 20 U/ml. At day 7, cells were harvested and tested for the presence of peptide-specific CD8⁺ T cells by an IFN- γ release ELISPOT assay.

ELISPOT assay

ELISPOT assay was performed using a commercially available kit (U-Cytech). Ninety-six-well polyvinylidene difluoride-backed plate was pre-coated with anti-IFN- γ mAb overnight at 4°C . The plate was blocked for 1 h at 37°C . PBMCs or murine splenocytes were dispensed at predetermined density in duplicate wells. A total of 20 $\mu\text{g}/\text{ml}$ peptide was added to stimulate the effector cells. The plate was incubated at 37°C for 24 h for human PBMCs or 40 h for murine splenocytes. Afterward, cells were removed and the plate was processed according to the manufacturer's instructions. The colored spots, representing epitope-specific IFN- γ -producing T cells, were counted using an automatic ELISPOT reader. Only brown colored spots with fuzzy borders were scored as spot-forming cells (SFC).

Tetramer preparation and staining

Tetrameric HLA-A*0201-peptide complexes (tetramers) containing P15 were constructed using the previously described method (46). Briefly, a DNA sequence containing a Bir A enzymatic biotinylation site was added to the COOH terminus of the HLA-A*0201 H chain or HLA-A*0201/H-2K^b chimeric H chain, which was a gift from A. Sewell (Nuffield Department of Medicine, The Peter Medawar Building of Pathogen Research, John Radcliffe Hospital, Oxford, U.K.), created by fusing human $\alpha 1$ and $\alpha 2$ domains to the H-2K^b mouse $\alpha 3$ domain. The entire construct was cloned into the pET-30a plasmid (Novagen) and transfected into competent *E. coli* for the protein expression. The purified recombinant HLA-A*0201 or HLA-A*0201/H2K^b chimeric H chain containing the Bir A site and human β_2m were refolded with peptide P15. The complexes were purified by chromatography using a Superdex G-75 size-exclusion column (Pharmacia) followed by Mono Q (Pharmacia) anion-exchange chromatography, and biotinylated using the Bir A enzyme (Avidity). The biotinylated complexes were tetramerized by mixing biotinylated HLA-A*0201-peptide or

HLA-A*0201/H-2K^b-peptide complexes and PE-labeled streptavidin (BioSource International) at a molar ratio of 4:1. Murine splenocytes or human PBMCs were incubated at 37°C for 30 min in staining buffer (PBS with 0.1% BSA and 0.1% sodium azide) containing the PE-labeled tetrameric complex. Cells were washed once with staining buffer and then incubated at 4°C in staining buffer containing saturating amounts of FITC-labeled anti-CD8 mAb (BD Biosciences) and PE-Cy5-conjugated anti-CD3 mAb (BD Pharmingen). Samples were detected by flow cytometry. Over 10⁶ events were acquired for each sample. Tetramer⁺ cells gated from CD3⁺CD8⁺ T lymphocytes were counted as epitope-specific CTLs.

Intracellular IFN- γ -staining assay

Intracellular cytokine staining was performed with the BD Biosciences Cytofix/Cytoperm Plus Fixation/Permeabilization kit (with BD Golgistop protein transport inhibitor containing monensin) according to manufacturer's instructions. In short, splenocytes from DNA vaccinated mice were respectively restimulated for 6 h in the presence of 20 μ M P15, P15'A (P15 without the last C-terminal amino acid), P15'B (P15 without the last N-terminal amino acid), SSp-1, S978, S1203. GolgiPlug containing monensin was added at a final concentration of 0.7 μ l/ml to block the intracellular transport processes. Anti-IFN- γ mAbs (BD Pharmingen; Biolegend) were added after cells were fixed and permeabilized using BD Biosciences Fixation/Permeabilization solution. After washing with PBS, stained cells were fixed with 0.5% paraformaldehyde and analyzed by flow cytometry.

Immunization of mice with peptide P15

HLA-A2.1/K^b Tg mice (47) were a gift from Prof. X. Cao (Institute of Immunology, Second Military Medical University, Shanghai, China). Tg mice were bred in a pathogen-free facility. Cell surface HLA-A2.1 expression was assessed by flow cytometry using FITC-labeled anti-HLA-A2 mAb BB7.2. Female Tg mice (6–8 wk old) were immunized s.c. at multiple sites with a mixture of peptides, IFA (Difco) (48), and the N-terminal fragment N333 (22–355 aa) of murine gp96 (49) three times weekly. The injection volume was adjusted to 200 μ l for each animal. Spleens were recovered 7 days after the last immunization, dispersed with a syringe plunger, and passed through a cell strainer. Erythrocytes were lysed with 0.83% ammonium chloride lysis solution. Splenocytes were then washed and resuspended. Specific CTLs were detected by ELISPOT assay and tetramer staining as mentioned previously.

Immunization of mice with DNA vaccine

Plasmid pTSh (50) with an insert of humanized SARS-CoV S-protein-encoding gene in which native codons were replaced with the degenerate

codons used most frequently in human genes was presented as a gift by Prof. H. Deng (Peking University, Beijing, China). Female HLA-A2.1/K^b Tg mice (6–8 wk old) were immunized with 60 μ g of plasmid DNA in 200 μ l of PBS (pH 7.4) at weeks 0, 3, and 6, respectively. Immune responses were measured 10 days after the final boost (23, 51).

Results

Selection of potential HLA-A*0201-binding peptides within SARS-CoV S and N proteins

Based on computer software predictions, 18 candidate nonameric and decameric peptides with the highest estimated half-time of dissociation from HLA-A*0201 were selected and synthesized (Table I). To evaluate the binding affinity of these peptides to HLA-A*0201 molecules, we used a peptide-induced stabilization assay of the HLA-A*0201 H chain and β_2 m in vitro. Among the 18 predicted peptides, 9 could refold with HLA-A*0201 H chain and β_2 m molecules with varying avidities (Fig. 1). Among these, P6, P10, P14, P15, and P17 had stronger avidities. To further investigate the binding affinity of these peptides to the HLA-A*0201 molecules at the cellular level, we used the T2 cell-peptide binding assay. The assay measures the increase of HLA-A*0201 molecules induced on T2 cells following exposure to exogenous HLA-A*0201-binding peptides. Increased detection of HLA-A*0201 is then extrapolated to the binding affinity of the corresponding test peptide. Of the 9 candidate peptides, only P7, P14, P15, and P17 were considered high-affinity ones (FI = 1.02, 1.26, 1.53, and 2.32, respectively), while the others all had a FI < 1. The positive control HBVC-1 bound HLA-A*0201 strongly (FI = 2.65), whereas no binding was associated with the negative control HBVC-2 (FI = 0.03) (Table I and Fig. 2).

Peptide-specific CTLs from PBMCs of SARS-recovered donors

PBMC samples obtained from 10 donors who had recovered from SARS were typed for HLA-A2 expression. The samples of four donors samples were HLA-A2 positive. Each of the nine candidate peptides from SARS-CoV S and N proteins were tested for their capacity to stimulate IFN- γ secretion in the PBMCs from the four

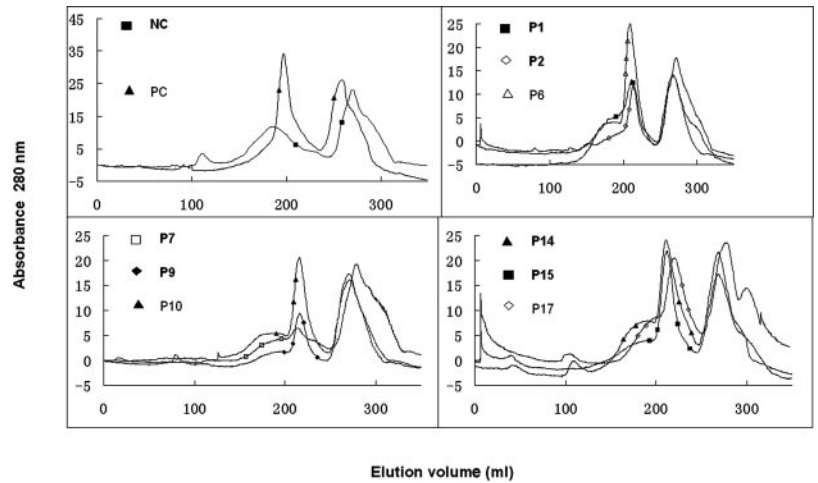
Table I. Predicted HLA-A*0201-restricted peptide epitopes for SARS-CoV spike and nucleocapsid proteins

Source	Name	Start Position	Sequence	Score ^a	FI ^b
SARS-CoV Nucleocapsid	P1	222	LLLLDRLNQL	309.050	0.64
	P2	113	YLGTPPEASL	98.267	0.47
	P3	331	WLTYHGAIKL	98.267	ND
	P4	159	VLQLPQGTTL	36.316	ND
	P5	219	ALALLLLDRL	24.075	ND
	P6	223	LLLDRLNQL	1267.104	0.44
	P7	227	RLNQLESKV	69.552	1.02
	P8	317	GMSRIGMEV	50.232	ND
SARS-CoV Spike	P9	1042	VVFLHVTYV	484.238	-0.06
	P10	82	RLQSLQTYV	382.536	0.33
	P11	58	VLNDILSRL	342.461	ND
	P12	897	VLYENQKQI	232.693	ND
	P13	1174	NLNESLIDL	201.447	ND
	P14	373	KLNDLCFSNV	1896.335	1.26
	P15	411	KLPPDFMGCV	1134.008	1.53
	P16	163	YISDAFSLDV	685.390	ND
	P17	1077	FVFNGTSWFI	640.903	2.32
	P18	1197	YVWLGFIAGL	623.743	ND
HBcAg	HBVC-1	18	FLPSDFPPSV	2309.961	2.65
	HBVC-2	87	SYVNTNMGL	0.003	0.03

^a Estimated half-time of dissociation ($T_{1/2}$) of HLA-A*0201 peptide complexes calculated using the website [http://bimas.dcrn.nih.gov/molbio/hla_bind/index.html].

^b FI = (Mean FITC fluorescence with the given peptide – mean FITC fluorescence without peptide)/(mean FITC fluorescence without peptide). FI \geq 1 represents high-affinity peptide; FI < 1 represents low-affinity peptides.

FIGURE 1. Refolding of HLA-A*0201 H chain and β_2m with computer-predicted candidate peptides of SARS-CoV S and N protein. The refolded complexes (pointed to by arrows), eluted with the expected molecular mass (45 kDa), were analyzed by fast protein liquid chromatography Superdex G75 gel-filtration chromatography. Positive control (PC) peptide HBVC-1 is an HLA-A*0201-restricted HBV core 18–27 (FLPSDFFPSV) and negative control (NC) peptide HBVC-2 is a non- HLA-A*0201-restricted HBV core 87–95 (SYVNTNMGL). Peptides P1, P2, P6, P7, P9, P10, P14, P15, and P17 could refold with the HLA-A*0201 H chain and β_2m .



HLA-A2⁺ SARS-recovered donors *ex vivo*. The PBMCs were stimulated for 7 days in the presence of individual peptide and 20 U/ml rhIL-2. The induction of IFN- γ secretion was revealed by the ELISPOT assay. As shown in Fig. 3, P15 significantly elicited specific IFN- γ -producing CTLs from the PBMCs of all HLA-A2⁺ SARS-recovered donors rather than HLA-A2⁺ healthy controls (61.0 ± 8.4 vs 6.0 ± 1.4 SFC/2 $\times 10^5$ PBMCs, $p < 0.001$), and such a T cell response was not observed from the PBMCs stimulated by the other eight peptides. The significant difference of IFN- γ -producing cell numbers between HLA-A2⁺ SARS-recovered donors and HLA-A2⁺ healthy controls was also observed by 24-h direct ELISPOT assay with fresh isolated PBMCs (29 ± 6.3 vs 3 ± 1.5 , $p < 0.001$). In addition, HLA-A2⁻ SARS-recovered donors had a similar level of SFC value as normal controls (data not shown). Consequently, HLA-A*0201/P15 tetramer was prepared and used to confirm the existence of P15-specific CTLs. Fresh PBMCs from HLA-A2⁺ healthy donors, SARS-recovered donors, and HLA-A2⁻ SARS-recovered donors were stained with HLA-A*0201/P15 tetramer and the proportion of P15-specific CD8⁺ T cells was detected by flow cytometry. The results showed that P15-specific CTLs were detected from all HLA-A2⁺ SARS-recovered donors at an average of 0.21% of CD3⁺CD8⁺ T cells. In contrast, these specific CTLs were not detectable from the PBMCs of all tested HLA-A2⁺ healthy controls and HLA-A2⁻ SARS-recovered donors (Fig. 4).

In vivo induction of peptide-specific CTLs in HLA-A2.1/K^b-Tg mice immunized with peptide P15

To determine the *in vivo* immunogenic potential of peptide P15, we immunized HLA-A2.1/K^b transgenic mice with peptide P15, using IFA and the N-terminal fragment N334 (22–355aa) of murine GP96 as an adjuvant (49). After three rounds of weekly *in vivo* stimulation, splenocytes from primed mice were tested for IFN- γ production and chimeric HLA-A*0201 tetramer staining. Peptide P15 was used as stimulator at a concentration of 20 $\mu\text{g/ml}$ in the ELISPOT assay. Bulk CTLs from Tg mice inoculated with peptide P15 demonstrated a strong IFN- γ production. In contrast, no specific reactivity could be detected in splenocytes generated from Tg mice immunized with IFA and N333 (Fig. 5A). Fresh splenocytes from peptide P15-immunized mice were stained with P15-specific chimeric tetramer and the proportion of peptide-specific CD8⁺ T cells was detected by flow cytometry. As shown in Fig. 5B, ~1.2% of the CD3⁺CD8⁺ T cells were chimeric for the tetramer-positive CTLs. Also, no specific staining was observed for splenocytes isolated from mice immunized with IFA and N333 alone. These results indicated that peptide P15-specific CTLs were induced in the HLA-A2.1/K^b-Tg mice immunized with P15 peptides.

Induction of peptide-specific CTLs responses in HLA-A2.1/K^b-Tg mice following DNA vaccination

To demonstrate whether the epitope is a naturally processed peptide and compare its immunogenicity with three newly identified

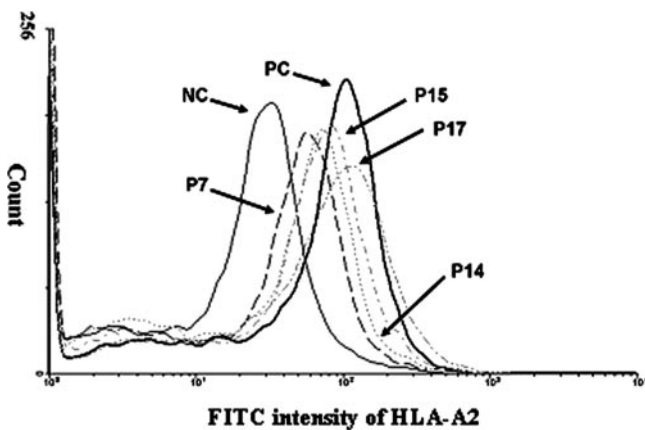


FIGURE 2. Stabilization of HLA-A*0201 molecule by T2 cell-binding assay. The fluorescence intensity of FITC-anti-HLA-A2 staining is shown with peptide P7, P14, P15, P17, positive control (PC) and negative control (NC) binding to T2 cells, respectively.

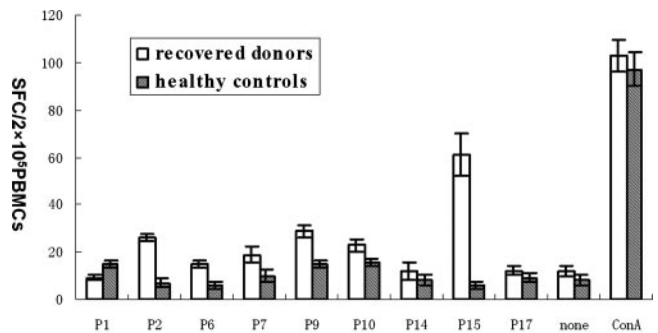
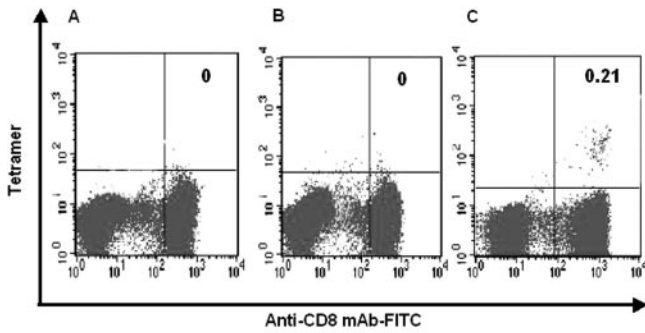


FIGURE 3. Identification of candidate peptides with the PBMCs of SARS-recovered donors by ELISPOT assay. The spots are a measure of IFN- γ secretion from PBMCs stimulated with various candidate peptides. \square , PBMCs from four donors who had recovered from SARS after 7–8 mo; \blacksquare , PBMCs from four HLA-A2⁺ healthy controls. Values are expressed as means \pm SD.



A. HLA-A2⁺ healthy donor
B. HLA-A2⁻ SARS-recovered donor
C. HLA-A2⁺ SARS-recovered donor

FIGURE 4. Measurement of P15-specific CTLs in HLA-A2⁺ the PBMCs of SARS-recovered donors by tetramer staining. P15-specific CTLs were measured from P15-peptide-stimulated PBMCs of HLA-A2⁺ healthy donors (A), HLA-A2⁻ SARS-recovered donors (B), and HLA-A2⁺ SARS-recovered donors (C), using PE-labeled HLA-A*0201/P15-tetramer along with PE-Cy5-labeled anti-CD3 and FITC-labeled anti-CD8 mAbs for cell staining. The numbers shown represent the percentage of tetramer⁺ cells within CD3⁺CD8⁺ T lymphocytes.

CTL epitopes of SARS-CoV spike protein (31, 32), we immunized HLA-A2.1/K^b-Tg mice with the plasmid pTSh encoding the S protein. After three i.m. inoculations, P15-specific chimeric tetramer was used to determine the frequency of peptide P15-specific CTLs in freshly isolated splenocytes. The results showed that 2.1% of CD3⁺CD8⁺ T cells were tetramer positive, while the positive cells were not detectable in Tg mice with empty plasmid inoculation (Fig. 6A). In parallel, a substantial quantity of IFN- γ -producing CTLs was detected by the ELISPOT assay and intracellular cytokine staining (Fig. 6, B and C), suggesting that a large number of peptide P15-specific CTLs were induced. These results suggest that the peptide P15 could be naturally processed *in vivo* and induce a specific CTL response. To determine whether P15 is a minimal stimulatory peptide, P15-derived nonameric peptides P15'A (KLPDDFNGC) and P15'B (LPDDFMGCV) were tested, and none of them could bind with HLA-A*0201 molecules in refolding and T2-binding assays (data not shown). In addition, they were proved unable to provoke obvious CTLs in splenocytes from pTSh-vaccinated Tg mice as detected by the ELISPOT and intracellular cytokine staining (Fig. 6, B and C). To compare the avidities of P15 and three previously identified CTL epitopic peptides of SARS-CoV, their ability to provoke CTLs in splenocytes from pTSh-vaccinated Tg mice was examined by IFN- γ release ELISPOT assay and intracellular IFN- γ staining. The ELISPOT showed that an average of 51, 25, 32, and 17 of IFN- γ -producing cells above background in 10⁵ splenocytes were elicited by stimulation of P15, SSp-1, S978, and S1203, respectively. The intracellular cytokine staining showed that 0.9, 0.4, 0.6, and 0.2% of IFN- γ ⁺ T cells in CD3⁺CD8⁺ T cells were observed by stimulation of P15, SSp-1, S978, and S1203, respectively (Fig. 6C). As a result, P15 showed the highest ability to provoke CTLs.

Discussion

It is well-established that optimal CTL responses require multiple antigenic epitopes for control of virus infections. In this study, we used an unusual approach to screen the potential CTL epitopes, i.e., starting from a computer motif prediction, followed by *in vitro* complex refolding and then came back to the usual T2-binding assay. We provide evidence for an HLA-A*0201-restricted, CD8⁺

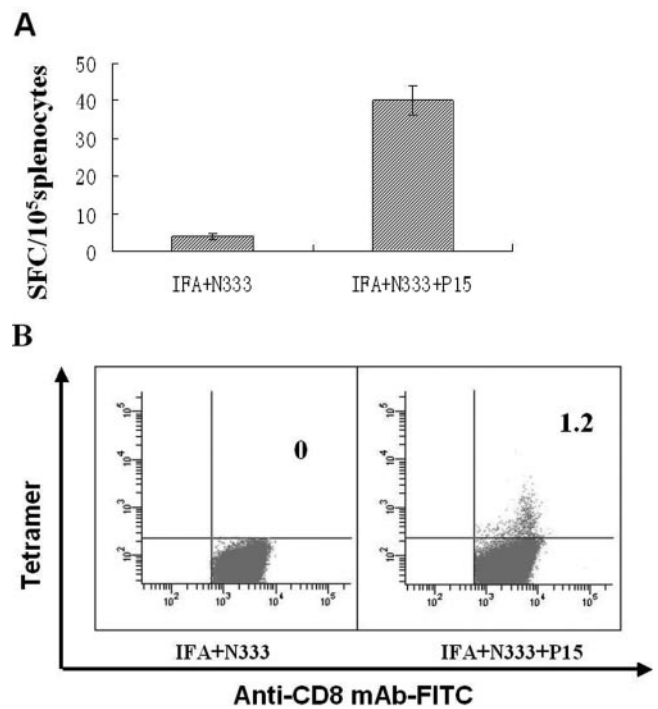


FIGURE 5. Detection of peptide P15-specific CTLs in peptide P15-immunized HLA-A2.1/K^b-Tg mice by ELISPOT assay and tetramer staining. In ELISPOT assay, splenocytes were incubated for 40 h in the presence of 10 μ M of peptide P15. Bars represent the mean number of SFC in 10⁵ splenocytes from three independent experiments (A). In tetramer staining, splenocytes were stained with PE-labeled HLA-A*0201/H-2K^b/P15 chimeric tetramer along with PE-Cy5-labeled anti-CD3 and FITC-labeled anti-CD8 mAbs. The numbers shown represent the percentage of tetramer⁺ cells within CD3⁺CD8⁺ T lymphocytes. The results are representative of three independent experiments (B).

T cell epitope specific for the SARS-CoV S protein. Eighteen 9- and 10-mer peptides derived from S and N proteins were selected for screening based on computer algorithms. Nine could bind with the H and L chains of HLA-A*0201 molecules with various avidities *in vitro* as determined by dilution refolding. They were further tested for their capacity to bind to HLA-A*0201 molecules on the T2 cell surface. It was observed that four of nine refoldable peptides were with high affinity. We observed that the affinity of these peptides was not entirely consistent as measured by the two assays. It has been suggested that the formation of MHC-I molecular complexes are affected by factors such as temperature and the conformation of the H chain of MHC class I, which may account for the discrepancy. To verify the results, we assayed cells from SARS-recovered donors to evaluate the CD8⁺ T effector cell-specific response to the nine candidate peptides. Only peptide P15-specific CTLs were significantly detected by stimulated ELISPOT and tetramer staining from four HLA-A2⁺ SARS-recovered donors. The immunogenicity of this peptide was thereby further studied in Tg mice. The results showed that P15-specific CTLs were generated in Tg mice both immunized by peptide P15 and an S gene-based DNA vaccine. Thus, we provide evidence that SARS-CoV S protein-derived peptide P15 is a novel, naturally processed HLA-A*0201-restricted CTL epitope. It is notable that P15 differs from the other three identified CTL epitopes SSp-1, S978, and S1203 in that it is a decameric epitope rather than a nonameric one and located within the angiotensin-converting enzyme 2 receptor-binding region of the S1 domain rather than the S2 domain.

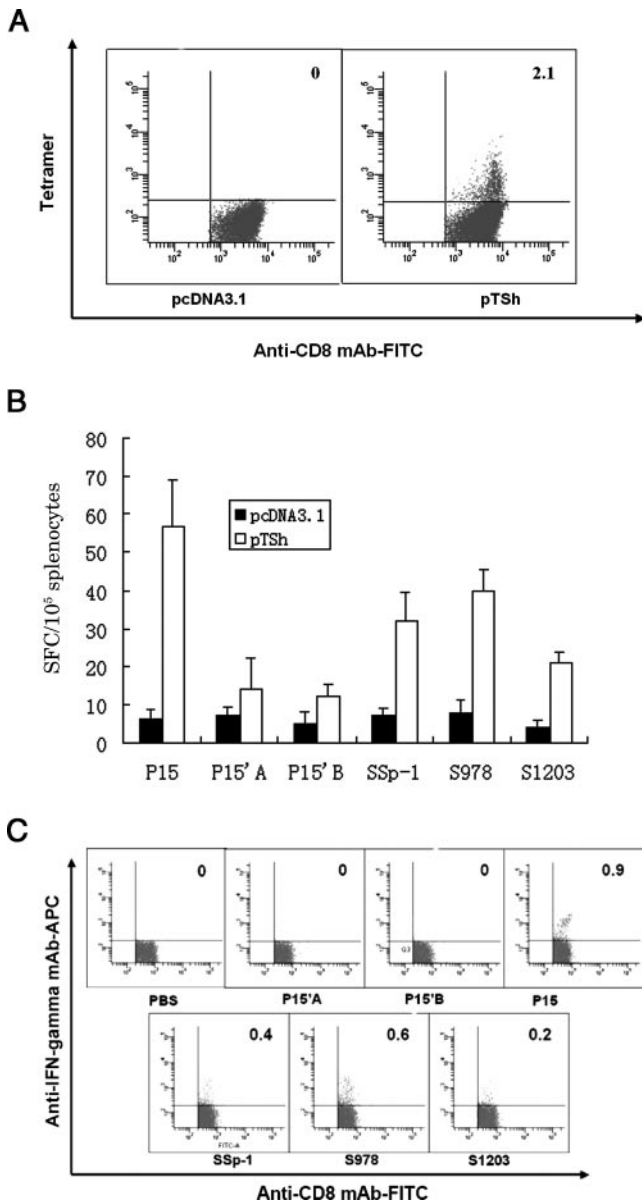


FIGURE 6. Detection of specific CTLs in DNA vaccine-immunized HLA-A2.1/K^b-Tg mice. Splenocytes from SARS-CoV S gene-based DNA-vaccinated mice were isolated for examination of epitopic peptide-specific CTLs. The splenocytes were stained with chimeric HLA-A*0201/H-2K^b/P15 tetramer along with PE-Cy5-labeled anti-CD3 and FITC-labeled anti-CD8 mAbs. Numbers indicate the percentage of tetramer⁺ cells within CD3⁺CD8⁺ T lymphocytes (A). The splenocytes were incubated for 40 h in the presence of 10 μ M individual peptide of P15, P15'A, P15'B, SSp-1, S978, or S1203. Bars represent the mean number of SFC from three independent experiments (B). The splenocytes were detected for intracellular IFN- γ expression with P15, P15'A, P15'B, SSp-1, S978, and S1203 as stimulator, respectively. Numbers indicate the percentage of IFN- γ ⁺ T cells within CD3⁺CD8⁺ T lymphocytes (C). The results are representatives of three independent experiments

After encountering viral Ags under conditions of appropriate costimulation, T cells proliferate and differentiate into IFN- γ -producing cytotoxic CD8⁺ T lymphocytes and cytokine-producing Th1 or Th2 effector CD4⁺ T cells (52–54). Ag-specific CD8⁺ T cells then undergo an abrupt contraction phase, in which ~90–95% of the effector cells are rapidly eliminated by apoptosis. The remaining Ag-specific CD8⁺ T cells resist apoptotic events and enter a memory pool (55, 56) where they provide enhanced pro-

tection of the host on re-exposure to the pathogen and act to prevent low-grade viruses from re-emerging (57). The SARS-CoV infects epithelial cells in the respiratory tract causing interstitial pneumonia and elicits strong immune responses (58). Memory or surviving effector CTLs against S978 and S1203 epitopes have been reported to be detectable from SARS-recovered donors 2 mo after the onset of disease (31). In this study, CTLs still existed in the donors 7–8 mo after SARS-CoV infection and were presumably from the memory CTL pool. This is consistent with the fact that no recrudescent SARS patients have been reported. The magnitude of the memory CTL pool after infection depends upon the pathogen, dose of infection, the specific epitopes analyzed (55, 56), as well as early inflammation and IFN- γ production following infection (59). Further studies are required to determine whether the memory CTL pool is affected by the therapeutic method of SARS treatment, e.g., the administration of glucocorticoids.

The SARS outbreak caused significant morbidity (~8500 cases) and mortality (774 deaths), and had an estimated economic impact of \$90 billion worldwide in U.S. dollars (60). Although SARS-CoV infection of humans has been contained through effective infection-control measures, resurgence is still a threat due to the presence of animal reservoirs (12). Consequently, several vaccines have been urgently developed (21–23). Studies of the immune response to coronaviruses suggest that both humoral and cellular immunity contribute to protection (61–64). Recently, existence of memory CTLs against SSp-1, S978, and S1203 epitopes was reported in SARS-recovered patients over 1 year postinfection, and dual roles of CTLs in control of virus replication and immunopathology of acute SARS-CoV infection were suggested (65). It has been proved that DNA vaccine encoding the SARS-CoV S protein may induce effective T cell and neutralizing Ab responses as well as protective immunity in a mouse model (23). In this study, the use of HLA-A2.1/K^b-Tg mice immunized with an S gene-based DNA vaccine was effective in producing HLA-A*0201-restricted CTL epitopes and resulted in a variety of special CTLs against SARS-CoV. Immunization with P15 and an adjuvant elicited a substantial quantity of specific cytotoxic CD8⁺ T lymphocytes that could be used to assess the function of cellular immunity in protection and clearance of SARS-CoV. Using the N-terminal fragment N334 of GP96 with IFA seems to be especially well-suited for this purpose (49). This immune method can also be practiced easily and effectively in testing immunogenicity of potential CTL epitopes.

Our results showed that decameric P15 is a minimal stimulatory epitopic peptide, for removal of a single amino acid from either N- or C-terminal remarkably reduced its avidity. Our results also showed that P15 had a superior avidity to three previously identified epitopic peptides in eliciting specific IFN- γ -producing CTLs from SARS-CoV S gene-based DNA-vaccinated Tg mice. However, whether P15-induced CTLs contribute to control of SARS-CoV replication needs to be further investigated.

In conclusion, our study demonstrates that *in vitro* refolding is an effective approach to identify HLA class I-restricted T cell epitopes. Starting from this method, we successfully identified a novel HLA-A*0201-restricted, immunogenic CD8⁺ T cell epitope derived from SARS-CoV S protein.

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Disclosures

The authors have no financial conflict of interest.

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