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HLA-A*0201 T-cell epitopes in severe acute respiratory syndrome (SARS) coronavirus nucleocapsid and spike proteins

Yeou-Ping Tsao [d], Jian-Yu Lin [a], Jia-Tsrong Jan [b], Chih-Hsiang Leng [c], Chen-Chung Chu [e], Yuh-Cheng Yang [e], Show-Li Chen [a,f,*]

[a] Department of Microbiology and Immunology, National Defense Medical Center, Taipei 114, Taiwan
[b] Institute of Preventive Medicine, National Defense Medical Center, Taipei 114, Taiwan
[c] Vaccine Research and Development Center, National Health Research Institutes, Taipei 115, Taiwan
[d] Department of Ophthalmology, Mackay Memorial Hospital, Taipei 104, Taiwan
[e] Department of Medical Research, Mackay Memorial Hospital, Taipei 104, Taiwan
[f] Graduate Institute of Microbiology, College of Medicine, National Taiwan University, Taipei 100, Taiwan

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Abstract

The immunogenicity of HLA-A*0201-restricted cytotoxic T lymphocyte (CTL) peptide in severe acute respiratory syndrome coronavirus (SARS-CoV) nuclear capsid (N) and spike (S) proteins was determined by testing the proteins' ability to elicit a specific cellular immune response after immunization of HLA-A2.1 transgenic mice and in vitro vaccination of HLA-A2.1 positive human peripheral blood mononuclearcytes (PBMCs). First, we screened SARS N and S amino acid sequences for allele-specific motif matching those in human HLA-A2.1 MHC-I molecules. From HLA peptide binding predictions (http://thr.cit.nih.gov/molbio/hla_bind/), ten each potential N- and S-specific HLA-A2.1-binding peptides were synthesized. The high affinity HLA-A2.1 peptides were validated by T2-cell stabilization assays, with immunogenicity assays revealing peptides N223–231, N227–235, and N317–325 to be the first identified HLA-A*0201-restricted CTL epitopes of SARS-CoV N protein. In addition, previous reports identified three HLA-A*0201-restricted CTL epitopes of S protein (S978–986, S1203–1211, and S1167–1175), here we found two novel peptides S787–795 and S1042–1050 as S-specific CTL epitopes. Moreover, our identified N317–325 and S1042–1050 CTL epitopes could induce recall responses when IFN-γ stimulation of blood $CD8^+$ T-cells revealed significant difference between normal healthy donors and SARS-recovered patients after those PBMCs were in vitro vaccinated with their cognate antigen. Our results would provide a new insight into the development of therapeutic vaccine in SARS.

Keywords: Nucleocapsid; Spike; CTL epitope; SARS virus; HLA-A*0201

Severe acute respiratory syndrome (SARS) is caused by a novel coronavirus—SARS-associated coronavirus (SARS-CoV). SARS-CoV is an enveloped positive-stranded RNA virus [1,2]. The four structural genes of SARS are S gene, encoding a spike glycoprotein which plays a role in cell–cell fusion; small membrane protein (E), an integral membrane glycoprotein (M), and the N gene encoding a nucleocapsid RNA-binding protein that may be associated with viral M protein [3]. Presumably, SARS-CoV infects cells through the S protein, which binds to cell surface receptor-angiotensin-converting enzyme 2 [4]. After this initial attachment, the viral E protein fuses with the plasma membrane of the host cell, and a cascade of intracellular events follows, including interaction between the M and N proteins [4]. Hence, these four structural proteins are major targets for developing anti-SARS drugs or vaccines. To date many efforts have been made to find a vaccine that can block SARS-CoV infection and eliminate its viral loads.

* Corresponding author. Fax: +886 2 2391 5293.
E-mail address: showlic@ha.mc.ntu.edu.tw (S.-L. Chen).

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Infectious bronchitis virus (IBV), a prototype of the Coronaviridae family, causes a highly contagious respiratory disease of chickens. IBV-specific CTLs are critical to play a role in the elimination of virus particles during acute infection and subsequent control of the infection in chickens [5,6]. Therefore, CTL may determine the role of immunity in controlling viral pathogenesis. In this project, for generating SARS-CoV CTL response, we chose to analyze the two structural genes-S and N for defining HLA-A*0201-restricted CTL epitope for the future vaccination. Human CTLs are specific for peptides presented in the context of major histocompatibility complex (MHC) molecules. Prior to presentation, peptides are generated in the cytosol by limited proteolytic fragmentation of all available antigens, translocated to the endoplasmic reticulum, specifically sampled by MHC molecules, and exported to the cell surface, where they await CTL scrutiny. Subunit vaccines that contain small synthetic peptides corresponding to minimal cytotoxic T lymphocyte (CTL) epitopes have been shown to be highly effective for the induction of strong, protective CTL-mediated immunity against infectious virus and tumor growth in murine models [7]. Hence, in this study, we mapped the HLA-A*0201 CTL epitopes of SARS-CoV N and S proteins. We used online database analysis to predict HLA binding peptides of SARS-CoV N and S proteins and validated by T2-cell binding assay [8]. Our results identified three N- and two S-specific HLA-A*0201 peptides. Moreover, our identified N317–325 and S1042–1050 CTL epitopes could induce recall responses when IFN-γ stimulation of blood $CD8^+$ T-cells revealed significant difference between normal healthy donors and SARS-recovered patients after those PBMCs were in vitro vaccinated with its cognate antigen.

Materials and methods

Peptide synthesis. To determine potential vaccine candidates, ten each potential 9-amino acid peptides of SARS-CoV N and S proteins were synthesized. In addition, the HLA-A2.1-binding peptide YMDGTMSQV (tyrosinase 369–377) and the HLA-A1-binding peptide EADPTGHSY (MAGE-1 161–169) [9] were tested positive and negative control, respectively. These peptides were synthesized by solid-phase strategies on an automated peptide synthesizer (Abimed AMS 422) using Fmoc chemistry. Peptides were analyzed by reverse-phase high-performance liquid chromatography (HPLC) and dissolved in dimethyl sulfoxide (DMSO) at 1–5 mg/ml, liquated, and stored at −70 °C.

T2-cell stabilization assay: stabilization of HLA-A2.1 on the surface of T2-cells by synthetic peptides. To determine whether synthetic peptides could bind to HLA-A*0201 molecules, peptide-induced HLA-A*0201 up-regulation on T2-cells was examined according to a protocol described previously [8]. To measure the relative amounts of HLA-A2.1-peptide complexes formed, 2×10^6 cells were incubated with 100 μg of each N or S protein peptide for 3.5 h at 26 °C in HBSS supplemented with 5% FBS, 5 μg/ml human β 2-microglobulin (Sigma), and 5 μg/ml brefeldin A. The various titrations of peptide for T2-cell binding assay were tested, and 100 μg of peptide was the approximate concentration for this assay (data not shown). The cells were then washed and incubated for an additional 2 h at 37 °C to allow the remaining peptide-free MHC molecules to denature, followed by indirect immunofluorescence staining for HLA-A2.1 expression with BB7.2 Ab and goat anti-mouse IgG (F(ab')2) FITC (Jackson ImmunoResearch, West Grove, PA), being fixed with 1% paraformaldehyde, and being analyzed by flow cytometry. Results were calculated as a percentage of HLA-A2.1 expression using the formula: (fluorescence intensity of experimental peptide binding to HLA-A2.1 − mock peptide binding to HLA-A2.1) × 100%.

Production of recombinant N protein. The recombinant N protein was expressed from pRSETA vector (Invitrogen, Carlsbad, CA, USA) in *Escherichia coli* system BL21(DE3)Gold (Stratagene, Cedar Creek, TX, USA) by DNA recombinant technology and purified by Ni–NTA resin as reported by Liu et al. [10].

Animals. HLA-A2.1 transgenic mice were purchased and imported from the Jackson Laboratory, and maintained in our Institute under specific pathogen-free conditions. The transgenic mice with C57BL/6 background that express a MHC-I molecule of human HLA-A2.1 have been described previously [11]. These transgenic mice received each N- or S-specific peptide (100 μg) or N protein (50 μg) with 30 μg CpG oligodeoxynucleotide (CpG ODN 1826) via intramuscular injection three times at one week intervals. One week after the last immunization, we mapped the CTL epitopes of N and S proteins by analyzing antigen-specific $CD8^+$ interferon-γ^+ (IFN-γ^+) double-positive cells in HLA-A2.1 transgenic mice with flow cytometry [12,13].

Intracytoplasmic cytokine staining and flow cytometry analysis. Splenocytes (2×10^6) from peptide vaccinated HLA-A2.1 transgenic mice or controls were incubated for 12 h with a 30 μg stimulator (each N or S synthetic peptide) for the detection of N or S peptide-specific $CD8^+$ T-cell precursors. Golgistop (PharMingen, San Diego, CA) was added into each well at 6 h before the cells were harvested. Cells were then washed twice in FACScan buffer and stained with fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD8b.2 (Ly-3.2) monoclonal antibody (PharMingen). Cells were subjected to intracellular cytokine staining using the Cytofix/Cytoperm kit according to the manufacturer's instructions (PharMingen). Phycoerythrin-conjugated rat anti-mouse IFN-γ monoclonal antibody was purchased from PharMingen. FACS analysis was performed on a Becton–Dickinson FACScan with CELLQuest software (Becton–Dickinson Immunocytometry System, Mountain View, CA).

*HLA-A*0201 typing.* Blood samples were collected in acid citrate dextrose tubes and transported within 24 h to the Immunohematology Reference Laboratory, Mackay Memorial Hospital (Taipei, Taiwan). All samples were tested for HLA-A, B, C serology (standard microlymphocytotoxicity technique) using Terasaki Chinese HLA-ABC 72-Well Trays (lots 2, 3, 3A, 3B) [14].

In vitro vaccination and IFN-γ assay. Samples were obtained with informed consent from Tri-Service General Hospital and Mackay Memorial Hospital, Taipei, Taiwan, and the study was approved by the institutional Human Investigation Review Committee. Human PBMCs were isolated, respectively from 5 normal and 5 full recovery patients of severe SARS-infection with HLA-A*0201 type by separation on Ficoll–Hypaque density gradients and re-suspended in T-cell medium [15]. These five recovery patients including one laboratory worker and four hospital nurses contracted SARS-CoV during the epidemic period on 2003 [16]. They were afflicted with SARS disease and hospitalized until full recovery. All of them were identified virus RNA in respiratory secretions. Antibodies against SARS-CoV were detected by SARS-specific IgG capture ELISA and end-point dilution Ab titers. The Ab titers were ≧1:16 and were observed in all recovered patients. No Ab to SARS-CoV was detected in any healthy donors. All patients' bloods were harvested over one year post-infection.

The isolated peripheral blood mononuclear cells were incubated on plastic dishes for 2 h. The non-adherent lymphocytes were aspirated, the adherent fraction was cultured in medium containing 1% polled human AB serum, recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) (1000 IU/ml), and recombinant human interleukin 4 (IL-4) (1000 IU/ml) (R and D Systems, Minneapolis, Minn.). One hundred micrograms of recombinant N protein or the combination of multiple S peptides was incubated with the adherent cells at 37 °C. Recombinant human interleukin 2 (IL-2) (10 IU/ml), recombinant human interleukin 7 (IL-7) (10 IU/ml), recombinant human interleukin 1-beta (IL-1β) (10 ng/ml), and recombinant human tumor necrosis factor-alpha (TNF-α) (10 ng/ml) were added every second days into antigen-vaccinated adherent