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Detecting specific cytotoxic T lymphocytes against SARS-coronavirus with DimerX HLA-A2:Ig fusion protein

Yue-Dan Wang*, Wei Feng Chen*

Department of Immunology, School of Basic Medical Sciences, Peking University Health Science Center, Beijing 100083, China

Received 9 June 2004; accepted with revision 7 July 2004

Abstract

To assess specific cytotoxic T lymphocytes (CTLs) against Severe acute respiratory syndrome (SARS)-coronavirus, a modified DimerX flow cytometry assay was performed with peripheral blood mononuclear cell (PBMC) from HLA-A2⁺ SARS-recovered donors at different time points post disease. CD8⁺ DimerX-S1203⁺ CTLs were detected in the PBMC from these donors up to 3 months after recovery. The percentages of CD8⁺ DimerX-S1203⁺ cells paralleled the numbers of interferon- γ -positive spots in an ELISPOT assay using the same antigenic peptide. In conclusion, DimerX-based flow cytometry staining may prove to be a real-time method to screen for CTL directed at epitopes from a newly identified virus.

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Keywords: SARS; ELISPOT; DimerX; HLA-A2; Immune response; Cytometry

Introduction

Severe acute respiratory syndrome (SARS) is a newly described viral disease that first appeared within the past 2 years [1,2]. The pathogenic agent is the SARS coronavirus (SARS-CoV) that represents a new strain of coronavirus. To date, there is no effective treatment currently involving supportive therapy while the presumed anti-viral immune response develops. This appears to consist of neutralizing Ab, as well as cytotoxic T lymphocytes (CTL) that can be detected after SARS infection. We have previously shown that a nona-peptide corresponding to a sequence of the S2 protein from SARS-CoV could induce interferon- γ -pro-

ducing lymphocytes using an ELISPOT assay [3]. DimerX HLA-A2:Ig fusion protein/peptide complex staining and evaluation via flow cytometry have shown CTL for specific viral epitopes [4,5]. In this study, we present evidence for the presence of anti-SARS-CoV-specific CD8⁺ T cells in peripheral blood mononuclear cells (PBMC) obtained from SARS-recovered donors using a SARS S2 protein nona-peptide derived DimerX HLA-A2:Ig fusion protein.

Materials and methods

Peptide synthesis

Two nona-peptides corresponding to sequences from a fragment of either the S2 protein of SARS-CoV (S1203 [FIAGLIAIV]) or the homologous sequences of HCoV-229e (H1121 [ISVVLIFVV]) were tested. Both of these nona-peptides were synthesized and purified to 99.5% (Peptide Synthesis Facility, Department of Chemistry,

* Corresponding authors. Department of Immunology, School of Basic Medical Sciences, Peking University Health Science Center, 38 Xueyuan Road, Beijing 100083, PR China. Fax: +86 10 82801436.

E-mail addresses: wangyuedan@hotmail.com (Y.-D. Wang), wfchen@public.bta.net.cn (W.F. Chen).

Hong Kong University of Science and Technology). Both nona-peptides showed a high affinity for the HLA-A2 molecule by database analysis [6], and this was validated by T2 binding assay [3].

Patients and sample collection

PBMCs were isolated from blood samples obtained from five donors who had recovered from SARS on August 26, 2003. These donors worked in the Peking University First Hospital and were infected with SARS-CoV between April 23 and April 27, 2003. All five of these donors are female, express HLA-A2⁺, and ranged in age from 20 to 23 years old. Two age-matched, female HLA-A2⁻ SARS-recovered donors and two HLA-A2⁺ healthy donors acted as the control donors in this study. Detection of SARS-CoV antibody in the recovered donors (independent to the expression of HLA-A2) was performed using a commercially available ELISA kit (EUROIMMUN Medizinische Labordiagnostika AG) (Table 1). All donors gave informed consent for the collection and testing of the PBMC samples and the protocol was approved and authorized by the Hospital Ethic Review Committee.

IFN- γ -release enzyme-linked immunospot (ELISPOT) assay

The ELISPOT assay to detect IFN- γ -secreting CD8⁺T cells was performed according to the method described previously [7]. Briefly, PBMCs (1×10^5) collected from the donors listed above were plated in triplicate in round-bottomed 96-well plates. The cells were incubated with 10 μ g/ml of either nona-peptide in 5% CO₂ at 37°C. After 18-h stimulation, the cells were thoroughly mixed and transferred onto flat-bottomed 96-well nitrocellulose plates (HA Multiscreen, Millipore, Bedford, MA, USA) coated with anti-IFN- γ mAb (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 -

secreted spots were detected using a commercial kit (MABTECH, Sweden) according to the instructions of the manufacturer. The colored spots identifying the cytokine-producing cells were counted using a dissecting microscope. The mean number of spots from the triplicate wells was calculated and expressed as the number of specific IFN- γ -secreting cells/ 1×10^5 cultured PBMC cells [8].

DimerX HLA-A2:Ig fusion proteins for the analysis of specific T cells for SARS virus

The complex of SARS-CoV-specific and HcoV-229e-specific nona-peptide with DimerX HLA-A2:Ig fusion protein (gifts from Mr. Zhang Jing, BD Biosciences, USA) was produced with a modified active peptide-loading by acid stripping following the standard protocol described in Application Handbook 1st Edition, BD Biosciences [9]. Briefly, nona-peptide concentration was calculated as noted below by the formula and dissolved at 20 mg/ml using DMSO (SIGMA, US) and then further diluted in sterile PBS to 2 mg/ml until use, was incubated BD DimerX reagent, and was then exposed to 5 volume equivalents of citrate-phosphate buffer (pH5.8) at 37°C for 1.5–2 h. The pH of the mixture then was adjusted to 7.4 and the mixture was incubated at 4°C for 36 h to allow the protein to refold. PBMCs from SARS patients or controls were blocked with human AB serum for 1 h before staining. The cells were suspended in PBS at a concentration of approximately 1 million cells per 50 μ l and incubated with 4 μ g loaded BD DimerX at 4°C for 1 h. After washing once with 200 μ l PBS, the cells were resuspended in 100 μ l PBS containing 1 μ g FITC-labeled anti-mouse IgG (BD Biosciences, US) and incubated at 4°C for 45 min. After washing twice with 200 μ l PBS, the cells were resuspended in 100 μ l PBS containing 1 μ g PerCP-conjugated antihuman CD8 at 4°C for 45 min. After washing twice with PBS, the cells were resuspended in

Table 1
The data of patients studied

Number	Gender	Age	HLA-A2	ELISA	DIMERX (%) ^a		DIMERX (%) ^b	
					H1121	S1203	H1121	S1203
1	Female	21	+	+	0	13	0.01	0.19
2	Female	23	+	+	0	12	0.00	0.25
3	Female	21	+	+	1	35	0.02	0.42
4	Female	23	+	+	0	25	0.01	0.33
5	Female	21	+	+	3	15	0.00	0.28
6	Female	21	-	+	0	0	0.02	0.00
7	Female	20	-	+	0	0	0.00	0.00
8 ^c	Female	22	+	-	2	1	0.00	0.02
9 ^c	Female	23	+	-	0	0	0.00	0.00

^a The number of colored spots per 10^5 cells in wells with ELISPOT assay.

^b The percentages of CD8⁺DimerX⁺ cells in CD8⁺ populations.

^c Two female and age-matched HLA-A2⁻ health donors who were not infected with SARS-CoV when the samples were collected.

0.5 ml of PBS for flow cytometric (FACSCalibur, Becton-Dickinson) analysis immediately.

Mp	(MdimerXRxDp)/Ddimer
Dp	molecular weight of peptide
Ddimer	50,000 Da
R	640 (recommended excess of molar ratio, BD Biosciences)
Mp	microgram peptide of interest
Mdimer	mg of BD DimerX in the reaction

Results and discussion

S1203 is a potent immunogenic T cell epitope in the S2 protein of SARS-CoV virus defined by our previous study [4]. S1203 is a nona-peptide with Leu and Val at positions 2 and 9 that anchor in the HLA-A2 peptide binding groove and show a similar high affinity to HLA-A2 molecules during T2 cell binding assay [4]. In PBMC from SARS-recovered donors, specific CD8⁺ CTLs for S1203 were identified by DimerX staining as well as with ELISPOT assay using this nona-peptide (Fig. 1). Furthermore, the results of DimerX staining paralleled the results of ELISPOT and both identify a class I MHC-restricted CD8⁺ T cell response (Table 1). In this study, PBMC from HLA-A2⁻ SARS-recovered donors and

healthy HLA-A2⁺ donors were also assayed and none of these controls responded in either assay system (Table 1). This suggests that DimerX HLA-A2:Ig is a useful tool in the screening of CTL directed against specific epitopes from the SARS-CoV proteins.

DimerX staining as well as Tetramer staining [4,10] provides a direct method using flow cytometry to identify the specific CD8⁺ CTL. Tetramer technology is technically difficult to produce and is useful for only one specific peptide. In contrast, DimerX can present a variety of antigenic peptides, which in this case were combined with HLA-A2. Thus, the DimerX-peptide complex technique can be easily adopted in a clinic immunology lab. The time course of anti-viral-specific CTL identified with these techniques can be relatively short following recovery from viral infection. This minimizes the utility of a complex technology such as a Tetramer because it requires identifying and preparing the appropriate antigen-specific epitope. In contrast, the DimerX:HLA-A2 fusion protein/S1203 method for staining antigen-specific C*+ CTL appears to be a readily adaptable method to detect viral-specific cells during the post-viral recovery phase. This method could prove to be valuable in understanding the normal immune response to SARS-CoV as well as potentially helping to explain the varied outcome associated with SARS infection.

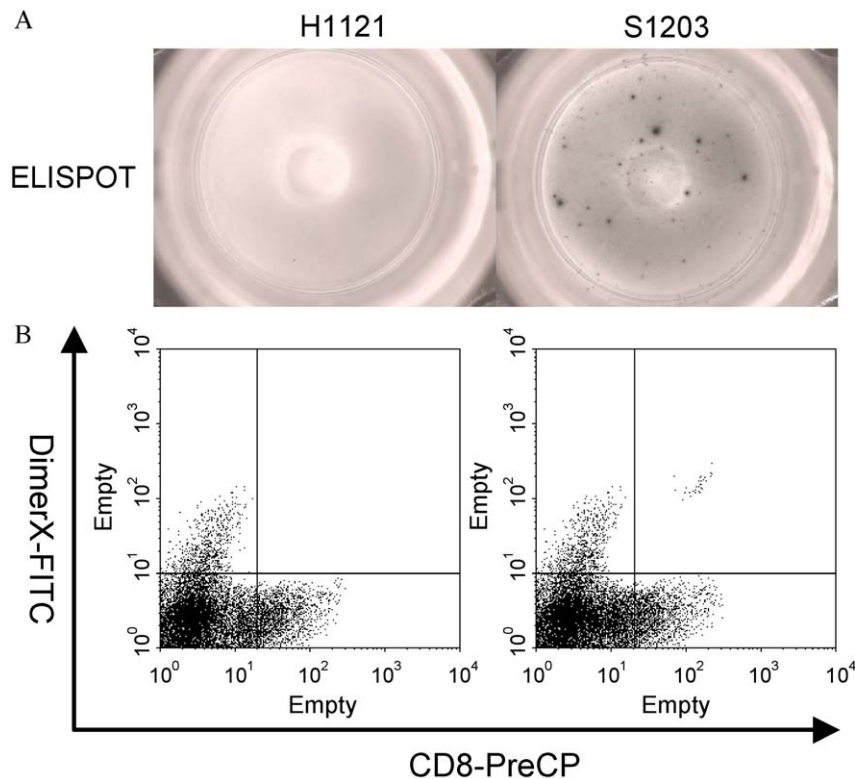


Fig. 1. Detection of anti-SARS-CoV-specific CTL. (A) The spots in ELISPOT assay with the PBMC obtained from HLA-A2⁺ SARS-recovered donors. PBMCs from SARS-recovered donors could be activated and secrete IFN- γ by S1203, but not by H1121. (B) CD8⁺DimerX-S1203⁺, but not CD8⁺DimerX-H1121⁺, cells were detected in PBMC from HLA-A2⁺ SARS-recovered donors.

Acknowledgments

The authors thank Mr. Zhang Jing, BD Biosciences, for providing DimerX:HLA-A2 infusion protein reagents. This work received a grant from the Ministry of Education, China and National Nature Science Foundation of China (NSFC, No. 30340011) and China Ministry of Science and Technology (2003CB514109).

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