Current HIV Research, 2007, *5*, 119-128

Identification of HLA-A11-Restricted HIV-1-Specific Cytotoxic T-Lymphocyte Epitopes in China

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Abstract: To fully define HLA-A11- restricted HIV-1- specific cytotoxic T-lymphocyte epitopes in China, a method combining the enzyme-linked immunospot (ELISPOT) assay with intracellular gamma interferon staining (ICS) of peripheral blood mononuclear cells (PBMC) was used to map the optimal epitopes targeted by ELISPOT and then to define the HLA restriction of epitopes by ICS. A novel HLA-A11-restricted CTL epitope and five other published HLA-A11-restricted epitopes previously identified by reverse immunogenetics or other methods were defined. The approach of integrating ELISPOT with ICS is both convenient and useful for the characterization of CTL responses to HIV-1 infection; this method is practical for defining novel epitopes and facilitates in developing new strategies for future vaccine design in China and other Asian countries.

Keywords: HIV, cytotoxic T-lymphocyte, epitopes, enzyme-linked immunospot (ELISPOT) assay, intracellular gamma interferon staining (ICS).

INTRODUCTION

Increasing evidence indicates that HIV-1-specific $CD8^+$ cytotoxic T lymphocytes (CTL) play a critical role in the control of viral replication in HIV-1 infection [30, 33]. The critical role of virus-specific CTL responses in controlling viremia has been directly demonstrated by $CD8^+$ T-cell depletion studies of simian immunodeficiency virus (SIV) infection in macaques, where $CD8^+$ T cells are necessary to effectively suppress viral replication [24, 34]. The HIV-1-specific $CD8^+$ T-cell responses generated during acute infection, in particular, appear to be critical determinants of the ultimate speed of progression to disease [2, 8]. Therefore, induction and maintenance of HIV-1-specific $CD8^+$ T-cell responses are considered a key elements in the development of effective HIV-1 vaccines [5, 6].

As the $CD8^+$CTL responses are restricted by polymorphic gene products of the MHC region, it is not surprising that certain class I HLA alleles are shown to be associated with resistance to HIV-1 infection in several cohorts of highly exposed persistently seronegative (HEPS) individuals at levels that are statistically significant [29, 36]. For example, a significant appearance of the class I HLA-A*1101 allele was observed in cohorts of HEPS female sex workers from Northern Thailand who were persistently HIV-1 seronegative, but seropositive for other sexually transmitted diseases [11, 28]. Although the ability of the host to resist HIV-1 infection is likely to depend on multiple factors, these cases of apparent resistance to HIV-1 infection suggest that HLA-A* 1101 may play a role in these processes. A better understanding of these interactions depends on the precise mapping of optimal CTL epitopes and defining the HLA class I restriction of these responses.

HLA-A*11 is one of the most common class I alleles in worldwide populations, ranging from 4 to 86% depending on the particular ethnic background [35] and is highly prevalent in Southeast Asia. In China, HLA-A*11 is one of the most frequently detected allele and HLA-A11 is the most common antigen type [37, 38]. Fifteen natural variants (-A*1101 to -A*1115) have been reported to date, with HLA-A*1101 being the most prevalent. Moreover, the position of the polymorphisms in several of these subtypes is unlikely to affect peptide binding [12]. Despite the fact that HLA-A11-restricted HIV-1-specific CTL epitopes have been identified in previous studies of Caucasian populations with HIV-1B infection and HEPS workers from Northern Thailand with HIV-1E infection [7, 15, 16], the systematic mapping of HLA-A11-restricted HIV-1-specific CTL epitopes is still in its infancy in China, a region of the world with a rapidly spreading HIV-1 epidemic.

Traditionally, CTL epitope mapping has relied on the long-term culture of CTL lines or clones and the subsequent use of chromium release cytolysis assays with target cells matched at individual HLA class I loci [9]. In the present study, to avoid the time-consuming effort and expense involved with culturing CTLs prior to defining epitopes and restricting alleles, a method combining ELISPOT with ICS of PBMCs was used to map the HLA -A11-restriction HIV-1-specific CTL epitopes.

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MATERIALS AND METHODS

Study Subjects

HIV-1-specific CTL responses were analyzed in detail in two patients. Individual CXA-031 was a Chinese Han whose precise date of infection is unknown, but at the time of study, his HIV infection had been documented to be exsistant for more than 6 years. He was observed to be antiretroviral therapy naïve, with his viral load and $CD4^+$ T Cell counts at the time of study were 157,000 RNA copies/ml of plasma and 192 cells/μl, respectively. Subject CXA-058 was also Chinese Han and had been HIV-1 infected since 1997. He received highly active antiretroviral therapy (two nucleoside reverse transcriptase inhibitors plus one nonnucleoside reverse transcriptase inhibitor) two years ago and was still receiving HAART. His viral load and $CD4^+$ T Cell counts at the time of study were < 500 RNA copies/ml of plasma and 611 cells/μl, respectively.

An additional eight HIV-1-infected individuals with HLA-A11 were screened for HIV-1-specific CTL responses against the defined HLA-A11-restricted CTL epitopes (Table **1**). All subjects in our study were infected with HIV-1 subtype B. In addition, all individuals gave informed consent for participation in the study.

HLA Typing and Subtyping

HLA class I molecular typing was performed at the Department of Molecular Biology in Nan-Kai University using sequence-specific primer PCR (SSP-PCR). HLA class I types of the subjects studied are shown in Table **1**.

Synthetic HIV-1 Peptides

Four hundred and twenty-five overlapping peptides (15- to 20-mers with 10-amino-acid overlaps) spanning the entire HIV-1 clade B Gag (p15, p17, and p24), Pol (Int, Prot, and RT), Env(gp120 and gp41), regulatory (Rev and Tat) and accessory (Vpr, Vpu, Vif, and Nef) protein sequences were synthesized on an automated peptide synthesizer (MBS 396; Advanced ChemTech, Louisville, Ky) using fluorenylmethoxycarbonyl chemistry. In addition, peptides corresponding to optimal HIV-1 CTL epitopes described for the individual's HLA class I type and truncated peptides for the fine mapping of novel optimal CTL epitopes were also synthesized on an automated peptide synthesizer (8~14-mer, Huachen company, Xi'an, *China*) using fluorenylmethoxycarbonyl chemistry. The purity of peptides in most cases was above 90%. These peptides (15~20-mer) were provided by Partners AIDS Research Center, Massachusetts General Hospital in Harvard Medical School.

Characterization of HIV-1-Specific CTL Responses by ELISPOT Assay

Fresh PBMC were separated from whole blood by Ficoll-Hypaque (Sigma) density gradient centrifugation and plated in 96-well polyvinylidene difluoride-backed plates (MAIP S45; Millipore, Bedford, Mass.), which had been previously coated with 100μl of anti-gamma-interferon (IFN-γ) MAb1-D1k (0.5 μg/ml; Mabtech, Stockholm, Sweden) overnight at 4°C. Peptides were added directly to the wells at a final 10μM concentration. Cells were added to the wells at 100,000 cells per well in a final volume of 130 μl of R10. For negative controls, 100,000 PBMC were incubated with R10 alone, without adding peptides. The plates were incubated at 37°C with 5% CO_2 overnight (14 to 16 h), then washed six times with phosphate-buffered saline (PBS) before 100μl of biotinylated anti- IFN-γ MAb 7-B6-1 (1 μg/ml; Mabtech) was added, and incubated at room temperature for 90 min. After being washed again with PBS, 100μl of 1: 20,000-diluted streptavidin-alkaline phosphatase conjugate (Mabtech) was added per well to the plate. The plates were incubated at room temperature for 45 min. Wells were again washed with PBS, and individual IFN-γ-producing cells were detected as dark spots after a 20- to 30-min color reaction with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium using an alkaline phosphatase-conjugated substrate (Bio-Rad Laboratories, Hercules, Calif.). Spots were counted by ELISpot reader (Autoimmun Diagnostika GmbH Ebinger straβ4. Germany) and were expressed as spot-forming cells (SFC) per 10^6 PBMC or per 10^6 input cells. The number of specific IFN-γ-secreting T cells was calculated by subtracting the negative control value from the established SFC count. Results of 40 or greater SFC/10^6 input cells were considered positive. Negative controls were always <40 SFC/10^6 input cells.

Table 1. HLA Types and Clinical Information of Study Subjects

Subjects	HLA types	Viral load (copies/ml)	$CD4^+$T cell count (cells/mm^3)	Treatment
CXA-019	A*11/26,B*52/40,CW*03/07	<500	518	None
CXA-020	A*02/11,B*15/38,CW*06/03	375,000	287	None
CXA-025	A*33/11,B*18/38,CW*07/12	101,0	135	None
CXA-029	A*11/11,B*58/58,CW*03/03	721,000	54	None
CXA-031	A*02/11,B*15/1502,CW*0410/0805	157,000	192	None
CXA-036	A*02/11,B*39/46,CW*01/07	<500	242	None
CXA-043	A*24/11,B*13/15,CW*03/04	<500	153	2NA+1NNRTI
CXA-057	A*02/11,B*27/4601,CW*01/1202	370,20	93	None
CXA-058	A*02/11,B*13/40,CW*03/06	<500	611	2NA+1NNRTI
CXA-068	A*29/11,B*0705/13,CW*03/15	<500	332	2NA+1NNRTI

NA, nucleoside reverse transcriptase inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor.

Fine Mapping of Optimal CTL Epitopes by ELISPOT Assay

For the fine mapping of the epitope by ELISPOT, the same peptide truncations were used as previously described in the chromium release assay [4, 18]. A total of 50,000 PBMC were incubated with concentrations from 10^{-4} to 10^{-11} M peptide overnight on the ELISPOT plate. All assays were run in duplicates. The optimal peptide was defined as the peptide that induced 50% maximal specific IFN-γ production by T cells at the lowest peptide concentration.

Intracellular IFN-γ Staining

ICS assays were performed as described previously [21]. Briefly, 1.0 x 10^6 PBMC were incubated with 4 µM peptide and anti-CD28 and anti-CD49d MAbs (each at 1 µg/ml; Becton Dickinson) at 37°C and 5% CO_2 for 1 h before the addition of brefeldin A (10 µg/ml; Sigma). Following further 6 h incubation at 37°C and 5% CO_2, the cells were placed at 4°C overnight. PBMC were then washed and stained with surface antibodies, anti-CD8 and anti-CD3 (Becton Dickinson) at 4°C for 20 min. Following washing, the PBMC were fixed and permeabilized (Caltag, Burlingame, Calif.) and anti- IFN-γ MAb (Becton Dickinson) was added. Cells were then washed and analyzed. For assays using HLA-matched or mismatched B-LCL, B-LCL that were pulsed with 10µM peptide for 1 h were washed thrice prior to incubation with PBMC at 10^5 BCL and 5 x 10^5 PBMC in 1 ml of R10 medium. The anti-CD28 and anti-CD49d MAbs were then added, and the assay was run exactly as described above.

RESULTS

HIV-1-Specific CTL Responses can be Rapidly Identified by ELISPOT Assay

In order to define CTL responses restricted by HLA-A*11, two subjects (CXA-031 and CXA-058) expressing this allele were evaluated. Characterization of HIV-1-specific CTL responses was performed for individuals CXA-031 and CXA-058 by using ELISpot and a panel of 425 overlapping 15-to 20-mer peptides spanning the full-length sequences of HIV-1 clade B. Subject CXA-031 had T-cell responses against 15 regions of HIV-1 proteins, including 7 responses against structural proteins, 5 responses against regulatory proteins and 3 against accessory proteins (Table. **2**). The magnitude of T-cell responses to the individual overlapping peptides ranged from 50 to 570 SFC/10^6 PBMC (mean, 176 SFC/10^6 PBMC). 38 overlapping peptides were targeted by T cells in subject CXA-058, including 31 responses against structural proteins, 2 responses against regulatory proteins and 5 responses against accessory proteins (Table. **3**). The magnitude of responses to individual overlapping peptides ranged from 90 to 760 SFC/10^6 PBMC (mean, 307 SFC/10^6 PBMC). The data above describes the regions within HIV-1 targeted by T cells in subjects CXA-031 and CXA-058 but does not indicate the number of epitopes contained within each region; some overlapping peptides may contain the same minimal epitopes in their overlap and a single overlapping peptide may contain more than one epitope [22]. In order to better determine the optimal epitope sequences, an ELISPOT assay using PBMC and serial dilutions of truncated peptides previously described was performed [20, 25]. As shown for subject CXA-058, strong responses were observed within the 18-mer peptide DIIATDIQTKELQKQITK (Pol residues 917 to 934). Using two 14-mer peptides that overlapped by 10 amino acids and together spanned the 18-mer Pol residues 917 to 934, it was determined that the optimal epitope was within the 14-mer DIIATDIQTKELQK (data not shown). By incubating PBMC in the ELISPOT assay with serial dilutions of the 9-mer IIATDIQTK (IK9) and serial dilutions of four additional peptides, which had 1 amino acid added to or deleted from the N- or C-terminal residues of the IK9 sequence, re-

Table 2. Responses Detected in ELISpot Assays of PBMC from Subjects CXA-031

Patient	Overlapping peptide	Amino acid sequence	magnitude of CTL responses (SFC/million PBMCs)
CXA-031	P17-4	GKKKYKLKHIVWASREL	70
	p17-11	TGSEELRSLYNTVATLY	120
	P17-12	SLYNTVATLYCVHQRIEV	120
	Nef -10	EVGFPVRPQVPLRPMTYK	110
	Nef -12	YKAAVDLSHFLKEKGGL	570
	Nef-13	SHFLKEKGGLEGLIYSQK	50
	Rev-2	DEELLKTVRLIKFLY	60
	Rev-3	KTVRLIKFLYQSNPPPS	60
	Tat-3	QPKTACTNCYCKKCCFH	70
	Tat -5	FHCQVCFTTKGLGISYGR	500
	Tat-6	TKGLGISYGRKKRRQRRR	60
	Pol-36	KKKSVTVLDVGDAYFSV	500
	Pol-46	RKQNPDIVIVQYMDDLYV	150
	Pol-57	NDIQKLVGKLNWASQIYA	60
	Pol-58	KLNWASQIYAGIKVKQL	50

Table 3. Responses Detected in ELISpot Assays of PBMC from Subjects CXA-058

Patient	Overlapping peptide	Amino acid sequence	magnitude of CTL responses (SFC/million PBMCs)
CXA-058	P17-5	KHIVWASRELERFAV	150
	P17-9	EGCRQILGQLQPSLQTGS	180
	P17-10	QLQPSLQTGSEELRSLY	190
	P17-11	TGSEELRSLYNTVATLY	130
	P24-9	GGHQAAMQMLKETINEEA	290
	P24-10	LKETINEEAAEWDRLHPV	150
	P24-11	AAEWDRLHPVHAGPIA	350
	P24-12	LHPVHAGPIAPGQMREPR	90
	P24-15	SDIAGTTSTLQEQIGWM	240
	P24-17	WMTNNPPIPVGEIYKRWI	100
	P24-18	PVGEIYKRWIILGLNKIV	90
	P24-21	SILDIRQGPKEPFRDYV	430
	P24-22	GPKEPFRDYVDRFYKTLR	90
	P24-24	LRAEQASQEVKNWMTETL	320
	P24-32	EAMSQVTNSATIMMQR	280
	P15-8	QMKDCTERQANFLGKIW	300
	P15-9	RQANFLGKIWPSHKGR	400
	P15-10	GKIWPSHKGRPGNFLQSR	140
	Nef-4	RRAEPAADGVGAVSRDL	350
	Nef-5	DGVGAVSRDLEKHGAI	330
	Nef-13	SHFLKEKGGLEGLIYSQK	460
	Rev-11	PLQLPPLERLTLDCNED	210
	Rev-14	TQGVGSPQILVESPAVL	760
	Pol-85	QKTELQAIHLALQDSGL	90
	Pol-86	IHLALQDSGLEVNIV	310
	Pol-103	LKGEAMHGQVDCSPGIW	410
	Pol104	GQVDCSPGIWQLDCTHL	340
	Pol121	AVFIHNFKRKGGIGGYSA	240
	Pol-122	RKGGIGGYSAGERIVDII	390
	Pol-123	SAGERIVDIIATDIQTK	350
	Pol-124	DIIATDIQTKELQKQITK	340
	Pol-131	KVVPRRKAKIIRDYGKQM	480
	Pol-132	KIIRDYGKQMAGDDCVA	280
	Vpr-7	ETYGDTWAGVEAIIRIL	270
	Vpr-8	AGVEAIIRILQQLFIHF	130
	Env26	YRLISCNTSVITQACPKV	490
	Env74	IVQQQNNLLRAIEAQQHL	650
	Env75	LRAIEAQQHLQLTVWGI	690

spectively, the 9-mer IIATDIQTK was demonstrated to be the optimal CTL epitope (Fig. **1**). The other optimal epitopes within overlapping peptides were defined by this method in subjects CXA-031 and CXA-058 (Table **4**).

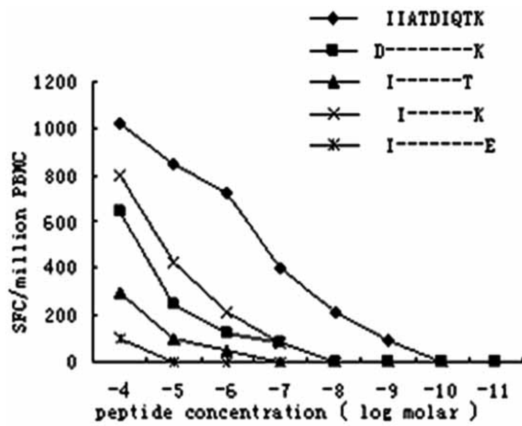


Fig. (1). Recognition of a novel HLA-A11-restricted CTL epitope in Int. The subject studied was CXA-058. Incubation of PBMC with peptide truncations of Int peptide IIATDIQTK in an ELISpot assay as shown.

Definition of HLA Restriction by ICS

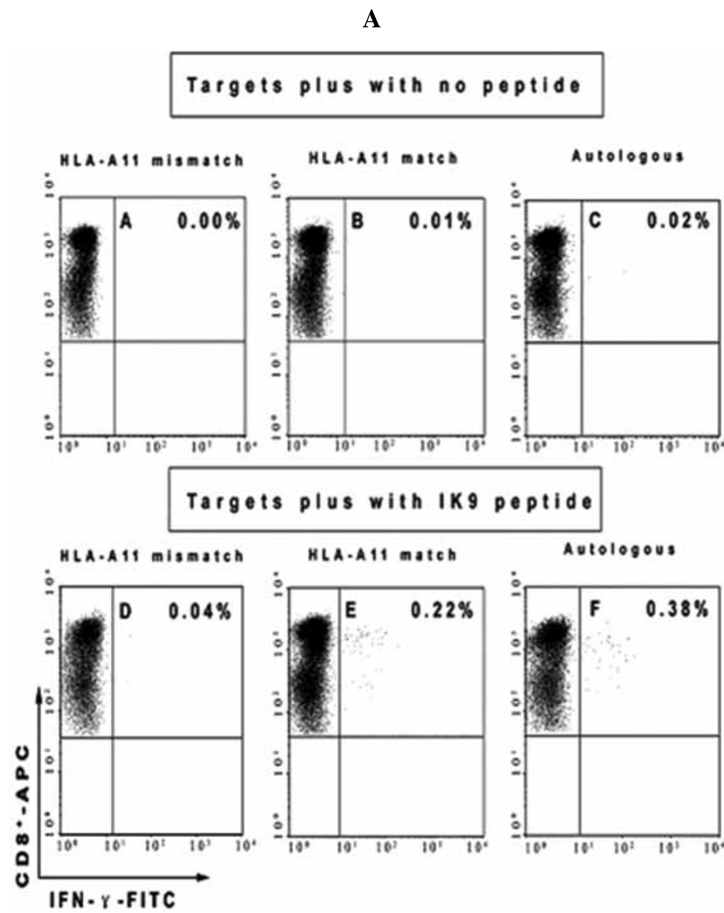
Previous studies have shown that optimal epitope sequences can be determined very rapidly and conveniently *via* ELISPOT using PBMC [4]. However, this approach has not been used to define the HLA restriction of the CTL response, since there is generally a high background of spot-forming cells in ELISPOT plates following incubation of PBMC with HLA-matched B-LCL even when B-LCL were not pulsed with peptide. To circumvent this problem, an approach was adopted to define HLA restriction *via* an intracellular IFN-γ staining assay [18]. A panel of Epstein-Barr virus-transformed B cell lines matched for various HLA class I alleles was pulsed with IK-9-mer peptide before co-incubation with CXA-058 PBMC. The following B cell lines were used: autologous CXA-058(A*02/11,B*13/40,CW*03/06),WSY (A*11/11,B*5401/1502,CW*08/01),XXL(A*02/32,B*35/44 ,Cw*04/04),MDL(A*26/30,B*13/15,CW*4/06),CXA-026(A *33/33,B*58/58,CW*03/03),WFX (A*24/31, B*51/40, CW*

Table 4. Optimal CTL Epitopes in CXA-031 and CXA-058

Subjects	Peptide location*	Amino acid sequence	HLA restriction of optimal CTL epitopes#
CXA-031	P17 77-85	SLYNTVATLY	A*2
	P17 84-91	ATLYCVHQR	A*11
	Nef 73-82	QVPLRPMTYK	A*11
	Nef 84-92	AVDLSHFLK	A*11
	Tat 36-45	VCFTTKGLGI	B*15
	Rt108-118	VLDVGDAYFSV	A2*
	Rt1179-187	VIVQYMDDL	A*2
CXA-058	P17 63-72	QLQPSLQTGS	A*2
	P17 77-85	SLYNTVATLY	A*2
	P24 61-77	GHQAAMQMLKE	A*2
	P24 70-78	KETINEEAA	B*4
	P24 78-86	AEWDRLHPV	B*4
	P24 87-101	HPVHAGPIAPGQMREPR	A*2
	P24 110-118	STLQEQIGWM	A*2
	P24 118-126	MTNNPPIPV	A*2
	P24 135-142	ILGLNKIV	B*27
	P24 152-162	DIRQGPKEPFR	B*27
	P24164-172	YVDRFYKTL	A*2
	P15 5-13	SQVTNSATI	A*2
	P15 64-71	TERQANFL	B*4
	P15 70-79	FLGKIWPSHK	A*2
	Nef 29-37	GVGAVSRDL	A*2
	Nef 92-100	KEKGGLEGL	B*4
	Rev 73-81	LQLPPLERL	A*2
	Rev 102-110	ILVESPAVL	A*2
	Rt 485-493	ALQDSGLEV	A*2
	Int 54- 61	QVDCSPGI Not described	
	Int 179-188	AVFIHNFKRK	A*11
	Int 196-203	GERIVDII Not described	
	Int 202-212	IIATDIQTK& A*11	
	Int 266-272	KIIRDYGK Not described	
	Vpr 53-63	TWAGVEAIIRI	A*11
	Vpr 59-67	AIIRILQQL	A*2
	Gp160 202-210	SVITQACPK	A*11
	Gp160 557-565	RAIEAQQHL	Cw*12

*Peptide location in reference virus strain based on HIV-1 HXB2 numbering system; # Based on Los Alamos National Laboratory HIV database; & Novel HLA-A11-restricted CTL epitope.

A



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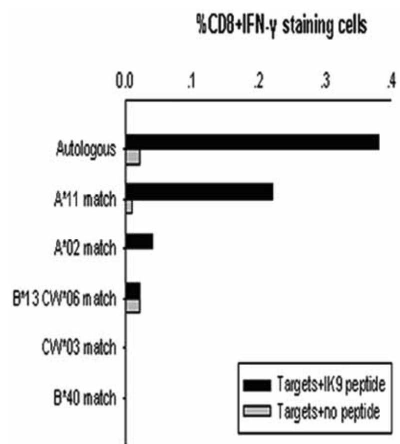

Fig. (2). HLA restriction of HLA-A11-restricted CTL responses by intracellular IFN-γ staining assay using effectors within PBMC. (**A**) HLA-A11 restriction of the IK9 response using PBMC from donor CXA-058 (A*02/11, B*13/40, Cw*03/06) and A11-matched B-LCL from donor WSY- PBMC (*A11/11, B*5401/1502, Cw*08/01). HLA-mismatched B-LCL were from donor XXL-PBMC (A*02/32, B*35/44, Cw*04/04). A: HLA-A11 mismatch targets plus with no peptide; B: HLA-A11 match targets plus with no peptide; C: Autologous targets plus with no peptide; D: HLA-A11 mismatch targets plus with IK9 peptide; E: HLA-A11 match targets plus with IK9 peptide; F: Autologous targets plus with IK9 peptide. (**B**) Determination of HLA restriction using CXA-058 PBMC in ICS assay targets either were pulsed with no peptide or were pulsed with IK9 (IIATDIQTK) peptide. The HLA class I types of the targets used were autologous CXA-058(A*02/11,B*13/40,CW*03/06),WSY(A*11/11,B*5401/1502,CW*08/01), XXL(A*02/32,B*35/44,Cw*04/04),MDL(A*26/30,B*13/15,CW*4/06),CXA-026(A*33/33,B*58/58,CW*03/03),WFX(A*24/31, B*51/40, CW*08/15). (Matching HLA class I alleles are shown in boldface).

08/15). Through this process, the HLA restriction of the response was determined unequivocally as HLA-A11 (Fig. **2A** and **2B**).

Definition of Five Further HLA-A11-Restricted CTL Epitopes

From the initial screening ELISPOT assays (Tables **2** and **3**), several other responses had been detected in PBMC from donor CXA-031 and CXA-058 using the sets of overlapping peptides, particularly in Gag /Nef/ Pol and Env. Optimization of 34 additional epitopes was determined by ELISPOT assays using PBMC (Table **4**); in each case, the HLA restriction of the response was determined by intracellular IFN-γ staining assay. Five other published HLA-A11-restricted epitopes previously identified by reverse immunogenetics or other method*s* were defined by ELISPOT and ICS in our study, they include P1784-91(TLYCVHQR), Nef73-82(QVP LRPMTYK), Nef 84-92(AVDLSHFLK), Int179-188(AVFI HNFKRK), Env202-210(SVITQACPK).

Frequency of Recognition of HLA-A11-Restricted HIV-1 CTL Epitopes and the Contribution of the A*11 Restricted Response in the Overall CD8 T Cell Responses

In order to determine the frequency of recognition of these HLA-A11-restricted CTL epitopes, especially novel epitope IK9, PBMC from ten HIV-1-infected subjects (including CXA-031 and CXA-058) with HLA-A11 were screened by ELISPOT (Table **5**). Six out of ten HLA-A11-positive subjects had positive CTL responses to one or more peptides. The number of peptides recognized per subject ranged from 0 to 3. The Nef epitope QVPLRPMTYK was recognized by four subjects and was the immunodominant HLA-A11-restricted epitope in the ten subjects tested. The novel epitope IK9 (Int 202-212 IIATDIQTK) was recognized by CXA-029 and CXA-058. Subjects CXA-031 and CXA-058 had broad responses to three epitopes. Other subjects had narrower responses, and CXA-019, CXA-020, CXA-025 and CXA-036 had no response to the peptides. Overall, although HLA-A11 is the most common antigen type in China [37, 38], the frequency of recognition of the HLA-A11-restricted CTL epitopes was lower in our study subjects.

In order to address how much CTL responses directed against HLA-A*11 restricted epitopes contributed to the total HIV-1-specific CTL responses, HIV-1-specific $CD^{+}8$ T cell responses were screened with overlapping peptides spanning the entire HIV-1 sequence from ten subjects with HLA-A*11 by ELISPOT. Magnitude of $CD^{+}8$ T cell responses against HLA-A* 11 restricted epitopes and peptides (containing HLA-A*11 epitopes) was determined and analyzed. Although HLA-A*11 restricted epitopes -specific $CD^{+}8$ T cell responses contributed importantly to the total CTL responses directed against HIV-1 in CXA-029 and CXA-031, which contributed more than 30% to total HIV-1-specific $CD^{+}8$ T cell responses, however, $CD^{+}8$ T cell responses directed against HLA-*A11 restricted epitopes contributed a mean of 13% to the total HIV-1 -specific CD8 T cell responses in ten subjects (Table **6**). Thus, CD8 T-cell response directed against HLA-*A11 restricted epitopes was not the lymain contribution to the total CTL responses directed against HIV-1.

DISCUSSION

Traditionally, identification of CTL epitopes is a difficult and labor-intensive process, especially the generation of CTL clones or peptide-specific lines. In our study, we provide a solution to this problem by combining the ELISPOT assay, which fine maps the epitope and ICS, determining HLA restriction. This method has the potential to define novel epitopes within 48 h of receiving fresh blood from a previously unstudied subject. In addition, the simplified process used here and the methods described previously [4, 18] have enabled the characterization of HIV-specific CTL responses to be performed in still developing countries.

Table 5. Frequency of Responses to HLA-A11-Restricted Epitope Peptides in Subjects with HLA-A11 Type

Subject	CTL response (SFC/million PBMC)					
	P17 83-91	Nef73-82	Nef84-92	Int179-188	Int202-212	Env202-210
	ATLYCVHQR QV	PLRPMTYK	AVDLSHFLK	AVFIHNFKRK	IIATDIQTK	SVITQACPK
CXA-019	0	0	0	0	0	0
CXA-020	0	0	0	0	0	0
CXA-025	0	0	0	0	0	0
CXA-029	0	0	0	160	210	0
CXA-031	350	600	850	0	0	0
CXA-036	0	0	0	0	0	0
CXA-043	0	90	0	0	70	0
CXA-057	0	140	0	0	0	0
CXA-058	0	0	0	240	340	490
CXA-068	0	50	0	0	0	0
Frequency	10%	40%	10%	20%	30%	10%

Table 6. Contribution of the A*11 Restricted Response in the Total HIV-1-Specific CD8 T Cell Responses

Subjects	magnitude of CTL responses induced by peptides (containing HLA-A*11 restricted epitopes) (SFC/million PBMC)	contribution of peptides (%)	magnitude of CTL responses induced by epitopes (SFC/million PBMC)	contribution of epitopes (%)
CXA-019	0	0	0	0
CXA-020	0	0	0	0
CXA-025	0	0	0	0
CXA-029	270	31	370	43
CXA-031	800	30	1800	67
CXA-036	0	0	0	0
CXA-043	140	11	160	13
CXA-057	120	3	140	4
CXA-058	1060	9	1070	9
CXA-068	60	16	50	13
Mean contri-bution		9		13

In the present study, a novel HLA-A11-restricted epitope was defined in subject CXA-058, IK9 (IIATDQTK). This peptide is part of the Int protein (Pol residues 918 to 934). Five other published HLA-A11-restricted epitopes previously identified by reverse immunogenetics or other methods were also defined by ELISPOT and ICS in our study, including P17 84-91(TLYCVHQR), Nef73-82(QVPLRPM TYK), Nef84-92(AVDLSHFLK), Int179-188(AVFIHNFK RK) and Env202-210(SVITQACPK). Two factors may have contributed to our definition of a novel HLA-A11-restricted epitope. First, a different method was used in the present study. The previous method of reverse immunogenetics [23] used the peptide-binding motif to predict and test potential peptide sequences. Subsequently, it identified binding peptides that are able to cause CTL recognition. While this is an efficient approach for epitope identification, the association between the peptide-binding motif and the amino acid sequence of the actual CTL epitopes is not always absolute [20, 25], as some epitopes may be lost. In addition, the previously used chromium release assay (CRA) may introduce bias in the CTL analysis, since the cells which easily expand *in vitro* are mainly memory T cells, while the activated effecter CTL which have proliferated *in vivo* for many cycles are more prone to apoptosis than to proliferate *in vitro* [31]. By combining enzyme-linked immunospot (ELISPOT) assays with intracellular gamma interferon staining (ICS), we have overcome the limitations described. The second factor, which may have contributed to our definition of the novel epitope is our study of a different ethnic population. In the present study, HLA-A11-restricted HIV-1-specific CTL response was studied in a Chinese population with HIV-1B infection, and not in a Caucasian population with HIV-1B infection or in the highly-exposed female sex worker population of Northern Thailand with HIV-1E infection as studied previously [7, 15, 16]. CTL responses depend on the predominant circulating virus and genetic background of the population [19], therefore, new HIV-1-specific CTL epitopes may be found in different ethnic populations.

HLA-A11 type was only defined in subjects in our study, which did not provide relevant detail on subtypes of HLA-A*11 alleles. Therefore, it must be considered that different HLA-A11 subtypes match different epitopes. However, fifteen natural variants (-A*1101 to -A*1115) have been reported to date, with HLA-A*1101 being the most prevalent. Moreover, the position of the polymorphisms in several of these subtypes is unlikely to affect peptide binding [12]. This suggests that our results of recognition of HLA-A11-restricted HIV-1 CTL epitopes were slightly influenced by different HLA-A11 subtypes.

China is a region with a prevalence of clade B/C or BC recombinants viral strains, with 90% subjects observed to be infected with HIV-1 subtype B according to the Los Alamos database [9]. We have identified the novel epitope IK9 (Int 202-212 IIATDIQTK) with the same amino acid sequence between the consensus B and C sequence. When held in comparison with the Shannon entropy score of the epitope IK9 in the 2004 alignment including clade B and C sequences published in the Los Alamos National Laboratory HIV Database [9], we found the Shannon entropy score to be lower than the rest of Pol integrase, which means the epitope IK9 is highly conversed between the two conscious sequences. The epitope may be used for vaccine development in China with a prevalence of clade B and C viral strains.

Among clade B-infected Caucasians, the HLA-A11 molecule can present at least two different epitopes each in Pol-RT (325-333 and 508-517) [26] and in Nef (73-82 and 84-92) [13], in spite of the HLA-A11 allele being not highly prevalent in Caucasians. In the Thai study, HLA-A11 was present in seven out of twelve Thai subjects. Of the 7 positive subjects, none recognized Pol-RT and only 2 recognized Nef [27]. Among the ten subjects with the HLA-A11 allele in our study, subjects CXA-031 and CXA-058 had broad responses to three epitopes. Other subjects had narrower responses, and four subjects had no response. Although the frequency of recognition of these HLA-A11-restricted CTL epitopes was higher in our subjects than in the Thai HEPS workers with HIV-1E infection, overall, recognition was

lower in our study subjects; on the other hand, CD8 T-cell response directed against HLA-*A11 restricted epitopes was not the main contribution to the total CTL responses directed against HIV-1. Other HIV-1-sepcific epitopes restricted by highly frequent HLA-I class antigen showed narrower CTL responses in populations with highly frequent this HLA-I allele. For example, HLA-A*02 is one of the most common class I alleles in worldwide populations. Day's study showed that the A2-restricted CTL response was found to be narrowly directed in most individuals expressing the HLA-A2 allele and in no case it was the dominant contributor to the total HIV-1-specific CTL response [14]. HIV adaptation against the most frequent HLA alleles of the host may explain this phenomenon. HIV may have evolved away from many responses in the past by mutating in positions 'allowed' by viral structural constraints. This may have left us with epitopes that, due to the some functional constraints, could not be escaped. This interpretation suggests that epitopes restricted by common HLA class I alleles have been widely eliminated because population-wide selection pressures prevent reversion of escape mutations. Alternatively, the epitopes may be targeted by functionally impaired CTLs, so there may be an incentive for the virus to change in those regions [1, 10, 32]. If this is the case, the 'common' epitopes identified to date may largely reflect ineffective epitopes, which may be less relevant for vaccine design. It is also possible that there are different CTL responses toward the autologous virus strain and the reference virus strain. Most epitopes identified in the past have been found using antigen sources based on selected reference strains of the virus. Thus, these can differ significantly from the autologous virus and bias the identification of epitopes toward relatively conserved regions of the viral genome [3]. Although many current studies use consensus sequences built from alignments that include contemporary strains, there is still an almost unavoidable bias towards the detection of relatively conserved CTL epitopes [17]. So peptides based on HIV-1 reference strains underestimate responses directed against the virus in more variable regions of the genome.

In conclusion, a novel HLA-A11-restricted CTL epitope and other five published HLA-A11-restricted epitopes previously identified by reverse immunogenetics or other methods were defined in HIV-1 by ELISPOT and ICS, which is a valuable advance in the approach to characterizing CTL responses. HLA- A11 is one of the most prevalent HLA class I molecules expressed in Chinese and other Asian populations, however, these CTL epitopes were not frequently recognized in HIV-1-infected individuals expressing HLA-A11. HIV adaptation to the most frequent alleles of its host and different CTL responses between the autologous virus strain and reference virus strain may explain this phenomenon. Because HLA allele frequencies vary greatly between ethnic populations, the frequency of HIV-1-specific CTL responses to individuals and the degree of recognition of epitopes may differ from one group to another even if the individual has been infected with the same HIV-1 clade. This is of critical importance in planning further vaccine trials in China and other Asian areas.

ACKNOWLEDGEMENTS

We thank Jian-ping Chen for assaying genetic subtypes of HIV-1 strains at National Center of Disease Control and Prevention and Shu Li for assaying HLA-I typing of subjects at Nan-Kai University. This study was supported in part by the National Institutes of Health (NIH Contract No. Nol-AL-30024) in U.S.A from the Foundation for AIDS & Immune Research.

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Received: July 3, 2006 **Revised: August 8, 2006** **Accepted: August 12, 2006**