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T cell receptor cross-recognition of an HIV-1 CD8+ T cell epitope presented by closely related alleles from the HLA-A3 superfamily

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Abstract

HLA-A3 and -A11 share similar peptide-binding motifs, however, it is unclear if promiscuous epitope presentation by HLA-A3 or HLA-A11 is associated with promiscuous TCR recognition. Here, we show that despite widespread cross-presentation of identical HIV-1 peptides in HIV-1-infected individuals expressing HLA-A3 or HLA-A11, peptides presented by HLA-A3 or HLA-A11 commonly exhibited clear immune distinctiveness with exclusive TCR recognition. Yet, using HLA-A3 and HLA-A11 tetramers for testing T cell cross-recognition of the HIV-1 Nef QK10 epitope, we observed in two study persons that specific CD8+ T cell populations were able to cross-recognize this peptide in the context of both HLA-A3 and HLA-A11. This cross-recognition was mediated by single cross-reactive TCRs, as shown by TCR sequencing in conjunction with TCR Vβ chain immunostaining. In each cross-reactive cell population, multiple TCR β chain variants were detected in the presence of only one TCR α chain variant. Thus, despite distinct TCR recognition of HLA-A3 or HLA-A11 presented HIV-1 peptides in the vast majority of cases, specific TCRs can cross-recognize their antigen in the context of both HLA-A3 and HLA-A11.

Introduction

TCRs recognize antigenic peptides presented by HLA class I or class II molecules. The set of HLA molecules expressed in a particular person is highly individual and allows each person to only present a specific pattern of foreign peptides to the immune system. Yet, specific HLA class I alleles have a high degree of sequence homology and share similar peptide-binding motifs, resulting in the presentation of identical epitopes, a fact for which they are referred to as HLA superfamilies (1–4). The impact of this promiscuous antigen presentation within one HLA superfamily on the recognition by TCRs, however, is currently not well understood. Previous data have shown that the specificity of TCRs for a given peptide-HLA complex is not entirely exclusive and that the same TCR can recognize different peptides presented by different HLA molecules (5–8). Whether promiscuous antigen presentation of identical peptides by HLA molecules within the same HLA superfamily is associated with promiscuous recognition by TCRs is currently unknown, but will be important for the understanding of the impact of HLA structure on the specificity of TCR recognition.

To date, nine major HLA class I supertypes have been classified according to similar peptide-binding motifs (9). HLA-A*0301, -A*1101, -A*3101, -A*3301 and -A*6801 alleles belong to the A3-like superfamily, which is defined by a shared preference for epitopes carrying A, L, I, V, M, S or T at position 2 and R or K at the C-terminus (2). The overall frequency of A3-like supertype alleles is very high (in the 37–53% range) and remarkably consistent in the major ethnic groups worldwide,

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although the distribution of the individual alleles of this superfamily varies significantly among different ethnic populations (1, 2). HLA-A3 and HLA-A11, the most frequent members of this superfamily, are extremely homogenous and differ only with regard to seven amino acid residues, most of them along the α1 and α2 helices (10). The presentation of identical epitopes by these closely related HLA molecules therefore provides a unique setting to analyze the impact of HLA amino acid sequence diversity on the selection of corresponding TCR clonotypes and the specificity of the TCR peptide-HLA class I interaction.

In this study, we analyzed cross-recognition of HIV-1 epitopes between the two major alleles from the HLA-A3 superfamily, HLA-A3 and HLA-A11, during natural HIV-1 infection and determined to what degree the promiscuous presentation of a frequently targeted HIV-1 epitope by HLA-A3 and HLA-A11 is associated with promiscuous, cross-reactive recognition by TCRs. Our data indicate that despite the very small sequence difference between HLA-A3 and HLA-A11, antigen presentation by these molecules appears to be strictly associated with distinct and exclusive TCR recognition in the vast majority of persons. In two study individuals, however, single TCRs were identified that were able to cross-recognize an HIV-1 epitope in the context of both HLA-A3 and HLA-A11. These data therefore represent a first step for the identification of structural components responsible for promiscuous antigen recognition by TCRs.

Methods

Subjects

A total of 63 HIV-1-infected individuals expressing HLA-A3 ($n = 40$), HLA-A11 ($n = 20$) or HLA-A3 and HLA-A11 ($n = 3$) were enrolled for this study. PBMCs of these study persons were used to test immune responses against all described HLA-A3/-A11-restricted optimal HIV-1 CD8+ T cell epitopes using enzyme-linked immunospots (ELISPOTs), or to analyze HLA-A3/-A11 cross-recognition of the HIV-1 QK10 peptide, or both, based on the quantity of available PBMC. Individuals were recruited from the Massachusetts General Hospital (MGH) or the Fenway Community Health Care Center in Boston. The MGH Institutional Review Board approved the study and each subject gave informed consent for participation in the study.

HLA typing

HLA class I molecular typing was performed at a commercial laboratory (Dynal Biotech, Oxford, UK) using standard procedures. Genomic DNA was extracted from whole blood PBMC samples using the Purgene DNA extraction kit according to the manufacturer's protocol.

Synthetic HIV-1 peptides

Twenty-three described HLA-A3- or HLA-A11-restricted optimal HIV-1 epitopes (9–11mers) were synthesized on an automated peptide synthesizer (MBS 396, Advanced ChemTech, Louisville, KY, USA) by using fluorenylmethoxycarbonyl chemistry.

Cell lines and media

EBV-transformed B-lymphoblastoid cell lines (BCLs) were established from PBMCs and maintained in R20 medium RPMI 1640 medium (Sigma, St Louis, MO, USA) supplemented with 2 mM L-glutamine, 50 U of penicillin ml^{-1}, 50 μg streptomycin ml^{-1}, 10 mM HEPES and 20% heat-inactivated FCS (Sigma) as previously described (11).

Measurement of peptide binding to HLA class I antigens

HLA class I molecules were purified from detergent lysates of EBV-transformed homozygous cell lines and used in a competitive binding assay with synthetic HIV-1 peptides containing 8–11 amino acids (12). Peptides known to bind to particular HLA antigens with high affinity were iodinated, using the chloramine-T method, and used as standards for binding assays. To measure HIV-1 peptide binding to HLA-3 or HLA-A11 molecules, 5–50 nM of the purified HLA molecules were incubated with the HIV-1 test peptides, at concentrations ranging from 120 μg ml^{-1} to 1.2 ng ml^{-1}, along with 1–10 nM of the radiolabeled standard peptides, for 48 h in PBS containing 0.05% NP40. All assays were run at pH 7 in a cocktail of protease inhibitors. Following incubation, HLA–peptide complexes were separated from free peptide by gel filtration on 7.8 mm × 15 cm TSK200 columns (TosoHaas 16215) with PBS pH 6.5 containing 0.5% NP40 and 0.1% NaN_3. The radioactivity in the column eluates was measured using a Beckman 170 radioisotope detector and the fraction of HIV-1 peptide bound was calculated.

ELISPOT assay

PBMCs were plated on 96-well polyvinylidene difluoride-backed plates (MAIP S45, Millipore, Bedford, MA, USA) that had been previously coated with 100 μl of an anti-IFN-γ mAb 1-D1k (0.5 μg ml^{-1}, Mabtech, Stockholm, Sweden) overnight at 4°C. Peptides corresponding to described optimal CTL epitopes were added directly to the wells at a final concentration of 14 μg ml^{-1}. Cells were added to the wells at 100 000 cells per well. The plates were incubated at 37°C, 5% CO_2 overnight (14–16 h) and then processed as described previously (13). IFN-γ-producing cells were counted by direct visualization and are expressed as spot forming cells (SFCs) per 10^6 cells. The number of specific IFN-γ-secreting T cells was calculated by subtracting the negative control value. Responses >50 SFC per 10^6 input cells after subtraction of background activity and higher than three times mean background activity were considered positive. The positive control consisted of incubation of 100 000 PBMCs with PHA.

Intracellular cytokine staining and flow cytometry

Assays were run using HLA-matched or -mismatched BCLs for the determination of HLA restriction of HIV-1-specific CD8+ T cell responses, as described previously (14): BCLs were pulsed with 4 μg ml^{-1} peptide for 1 h and subsequently washed four times prior to incubation with effectors at an E:T ratio of 5:1. One microgram per milliliter of anti-CD28 and anti-CD49d mAbs (Becton Dickinson) were then added. After incubation at 37°C, 5% CO_2, for 1 h, cells were mixed with 10 μg ml^{-1} Brefeldin A (Sigma). Following a further 4-h incubation, the cells were placed at 4°C overnight. After surface

marker labeling with anti-CD8 and -CD3 antibodies (Becton Dickinson), cells were fixed and permeabilized using the Caltag Fixation/Permeabilization Kit (Caltag, Burlingame, CA, USA) and anti-IFN-γ mAbs (Becton Dickinson) were added. Cells were analyzed on a FACSCalibur Flowcytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Control conditions were established by the use of BCLs that were unlabeled with peptide, but had otherwise been treated identically.

Sorting of tetramer+ HIV-1-specific CD8+ T cell populations

Fresh or frozen PBMC samples were stained with allophycoerythrin (APC)- or R-phycoerythrin (PE)-labeled MHC class I tetramers refolded with epitopic HIV-1 peptides (Beckman Coulter) and fluorophore-labeled CD8+ antibodies. Tetramer+ CD8+ cells were decontaminated (1% fixation solution A, Caltag) and sorted on a FACS Aria cell-sorting instrument (BD Biosciences) at 70 pounds per square inch. Electronic compensation was performed with antibody-capture beads (BD Biosciences) stained separately with individual antibodies used in the test samples. The purity of sorted cell populations was consistently >98%.

TCR α and β chain sequencing

mRNA was extracted from at least 1000 tetramer+ CD8+ T cells using the RNA easy mini kit (Qiagen, Valencia, CA, USA). Anchored reverse transcription (RT)–PCR was then performed using a modified version of the switching mechanism at 5′ end of RNA transcript (SMART) procedure and a TCR α and β chain constant region 3′ primer to obtain PCR products containing the Vα/β chain in addition to the CDR3 region, the Jα/β region and the beginning of the Cα/β region (15). Briefly, RT was carried out at 42°C for 90 min with primers provided for the 5′-rapid amplification of CDNA ends (RACE) reaction in a SMART–RACE PCR kit (BD Biosciences). First and second round PCRs were then performed using universal 5′-end primers and nested gene-specific 3′-end primers annealing to the constant region of the TCR α and β chain. PCR conditions were as follows—first run: 95° for 30 s and 72° for 2 min for five cycles; 95° for 30 s, 70° for 30 s, 72° for 2 min for five cycles and 95° for 30 s, 60° for 30 s and 72° for 1 min for 25 cycles; second run: 95° for 30 s, 60° for 30 s and 72° for 1 min for 30 cycles. When indicated, a panel of 21 different Vα chain-specific primers were used in combination with Cα outer/inner primers to amplify potential sub-dominant TCR α chain variants (16). The PCR product was ligated into the TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA) and used to transform *Escherichia coli* (Mach 1, Invitrogen). At least 20 colonies were selected, amplified by PCR with M13 primers, and sequenced by T7 or T3 primers on an ABI 3100 PRISM automated sequencer. Sequences were edited and aligned using Sequencher (Gene Codes Corp., Ann Arbor, MI, USA) and Se-Al (University of Oxford, Oxford, UK) and compared with the human TCR genes database (http://imgt.cines.fr:8104/home.html). The TCR clonotype composition remained consistent in repeated RT–PCR procedures using the same mRNA sample. The TCR Vα and Vβ chain classification system of the international ImMunoGeneTics database (17) was used throughout the entire manuscript.

Tetramer dissociation assays

CD8+ T cell avidity was analyzed using tetramer dissociation assays according to a recently published protocol (18). PBMCs suspended in staining buffer (PBS containing 1% FCS and 0.2% sodium azide) were stained with PE- and APC-labeled tetramers (Beckmann Coulter, Fullerton, CA, USA) refolded with epitopic HIV-1 peptides and FITC-labeled CD8 antibodies (BD Biosciences) for 45 min at room temperature, and then washed three times with staining buffer. To observe the dissociation of the tetramer, cells were re-suspended in staining buffer with unlabeled tetramers at a 100-fold surplus concentration. At selected time points, ~0.5×10^6 cells were withdrawn and fixed in 150 μl 2% PFA/PBS. Cells were then analyzed on a FACSCalibur Flowcytometer (Becton Dickinson). The MHC class I tetramer dissociation analysis was based on the total fluorescence of the tetramer+ population (19). Briefly, the total fluorescence of the gated CD8+ MHC class I tetramer+ population was calculated and normalized per gated lymphocyte. This total antigen-specific fluorescence of the CD8+ tetramer+ cell population was then normalized to the total fluorescence at the initial time point and the graphs were plotted on a logarithmic scale.

Statistical analysis

Statistical analysis and graphical presentation was done using the Prism software package. Results are given as mean ± SD or median with range. Statistical analysis of significance (*P*-values) was based on two-tailed *t*-tests and chi-squared test.

Results

Recognition of identical HIV-1-specific CD8+ T cell epitopes in individuals expressing either HLA-A3 or HLA-A11

HLA-A3 and HLA-A11 both belong to the HLA-A3 superfamily and share a common peptide-binding motif (2). Here we assessed the recognition of 23 described optimal HIV-1 CD8+ T cell epitopes restricted by HLA-A3 or HLA-A11 using a sensitive *ex-vivo* IFN-γ ELISPOT assay in 57 HIV-1-infected individuals (34 individuals expressing HLA-A3, 20 individuals expressing HLA-A11 and three individuals expressing both HLA-A3 and HLA-A11). Out of the 23 peptides, 17 were recognized both in the group of HLA-A3- and in the group of HLA-A11-expressing individuals (Table 1). In contrast, two epitopes were recognized exclusively by persons with HLA-A3 background, while four additional epitopes were only recognized by carriers of the HLA-A11 allele (Table 1).

In five of the 23 epitopes recognized in individuals expressing either HLA-A3 or -A11, we observed significant differences in the frequency of recognition. The epitopes RLRPGGKKK (RK9-p17) ($P < 0.01$), KIRLRPGGK (KK9-p17) ($P < 0.05$) and RMRGAHTNDVK (RK11-RT) ($P < 0.025$) were significantly more frequently recognized in carriers of HLA-A3, while the epitopes QIIEQLIKK (QKK9-RT) ($P < 0.001$) and IYQEPFKNLK (IK10-RT) ($P < 0.05$) were significantly more frequently targeted in individuals expressing HLA-A11.

To determine if the divergent frequency of recognition of specific epitopes in individuals expressing HLA-A3 or HLA-A11

Table 1. HLA binding affinity and frequency of recognition of the 23 epitopic peptides tested[a]

Epitope (protein)	HLA binding affinity (IC 50, nM)		Frequency of epitope recognition (%)		
	HLA-A3	HLA-A11	All study persons ($n = 57$)	A3 study persons ($n = 34$)	A11 study persons ($n = 20$)
QK10 (Nef)	23	11	46	35	60
AK9 (RT)	2	6	32	29	40
RK9 (p17)	21	11259	32	44	5
KK9 (p17)	820	>16260.16	30	38	10
AK9 (Nef)	30	6	23	18	35
RK11 (RT)	57.14	337.20	21	32	5
HK9 (Vif)	7	596	21	29	10
QKK9 (RT)	53.25	19.69	19	6	45
QVK9 (RT)	18	13	16	18	15
IK10 (RT)	563.78	130.17	16	9	30
ER10 (Rev)	409	114	14	18	10
RK10 (Vif)	16	128	12	18	5
GK9 (RT)	19	18	12	9	20
TI9 (p17)	2319.04	13202.67	11	9	15
TK10 (gp120)	5.46	3.43	11	15	5
AK11 (RT)	464.81	77.99	5	0	10
KK11 (Vif)	18	64	5	9	0
KK10 (RT)	23	82	5	9	0
GK9 (p24)	3246.42	2177.49	5	3	5
RR11 (gp41)	18	499	21	3	5
AK11 (p24)	9469.69	1440.62	4	0	10
SK9 (gp120)	6563.85	2560.98	19	0	5
AK10 (Int)	18	11	2	0	5

[a]Gray rows highlight epitopes with significantly different recognition among carriers of HLA-A3 or HLA-A11.

was related to differences in the binding affinity of epitopic peptides to either HLA-A3 or HLA-A11 molecules, we assessed the binding avidity for the tested peptides to both MHC class I molecules. Overall, we observed that the frequency of peptide recognition in HLA-A3-expressing individuals was correlated to the corresponding peptide binding affinity to HLA-A3 ($R = 0.4$, $P = 0.05$). In contrast, no correlation between the frequency of peptide recognition within HLA-A11-expressing individuals and the peptide binding affinity to HLA-A11 was found ($R = 0.17$, $P = 0.4$). Moreover, the three peptides that were significantly more frequently recognized in carriers of HLA-A3 had substantially enhanced binding affinity to HLA-A3 molecules compared with HLA-A11 molecules (mean of IC_{50} of 299.4 nM versus 9285.5 nM). In addition, the two peptides with preferential recognition in the HLA-A11-expressing individuals had considerably higher binding affinity to HLA-A11 compared with HLA-A3 (mean of IC_{50} of 75 nM versus 308 nM) (Table 1). Taken together, these data show that the majority of HIV-1 epitopes described for the HLA-A3 superfamily are recognized in both individuals expressing HLA-A3 and HLA-A11. However, epitopic peptides with enhanced binding affinity to HLA-A3 or HLA-A11 molecules were preferentially recognized in carriers of the respective alleles.

The HIV-1-specific CD8+ T cell epitope that was most frequently recognized by individuals expressing either HLA-A3 or -A11 was the $Nef_{73–82}$ epitope QVPLRPMTYK (QK10), which was targeted by 12 out of 34 individuals expressing HLA-A3, by 12 out of 20 individuals expressing HLA-A11 and by two out of three study persons expressing both HLA-A3 and HLA-A11. We subsequently focused our studies on this frequently targeted epitope (20, 21).

Infrequent natural occurrence of HLA-A3/-A11 cross-reactive QK10-specific CD8+ T cells

We next determined to what degree the promiscuous presentation of the QK10 peptide by HLA-A3 or HLA-A11 is associated with promiscuous recognition of the corresponding peptide-HLA complexes by cross-reactive TCRs. Using distinctively labeled HLA-A3 and HLA-A11 tetramers refolded with the QK10 peptide in a flow cytometric assay, we found that out of 25 HIV-1 infected study individuals with a detectable QK10-specific CD8+ T cell response (16 HLA-A3, 7 HLA-A11, 2 HLA-A3 and HLA-11), only two HLA-A3-expressing persons had CD8+ T cells able to bind to the QK10 peptide in the context of both HLA alleles (Table 2 and Fig. 1B). In contrast, in all other study persons, QK10-specific CD8+ T cells exclusively recognized their target peptide when presented by the exactly matching HLA class I allele (Fig. 1A). Interestingly, in the two study persons expressing both the HLA-A3 and the HLA-A11 allele, no HLA-A3/-A11 cross-reactive QK10-specific CD8+ T cells were observed, but distinct populations specific for the HLA-A3 or -A11 presented epitopes co-existed (Fig. 1A). These results obtained by flow cytometric analysis with distinctively labeled tetramers were confirmed by measuring antigen-specific IFN-γ secretion following stimulation with HLA-A3- or HLA-A11-expressing heterologous BCL pulsed with the QK10 peptide (Fig. 1C). Taken together, these data show that the majority of HIV-1-infected individuals expressing either HLA-A3 or HLA-A11 recognize the QK10 epitope only if presented by the exactly corresponding HLA molecule and that CD8+ T cells able to cross-recognize this peptide in the context of HLA-A3 and HLA-A11 are very rarely primed during natural HIV-1 infection.

Table 2. Recognition of HLA-A3/QK10 and HLA-A11/QK10 tetramers in the study cohort

T cell populations specific for	A3+ individuals $n = 16$	A11+ individuals $n = 7$	A3+/A11+ individuals $n = 2$
A3-QK10 only	14	0	2
A11-QK10 only	0	7	2
Both A3- and A11-QK10	2	0	0

Clonotypic composition of A3/A11 cross-reactive QK10-specific CD8+ T cells

As described above, in two of the HLA-A3-expressing study persons (AC-06 and AC-15; Table 4), QK10-specific CD8+ T cells consisted of two distinct populations: a small group of cells with exclusive binding to the HLA-A3/QK10 tetramer and a larger population binding both to the HLA-A3/QK10 and the HLA-A11/QK10 tetramers (Fig. 1B). To determine to what extent these two different populations varied with regard to

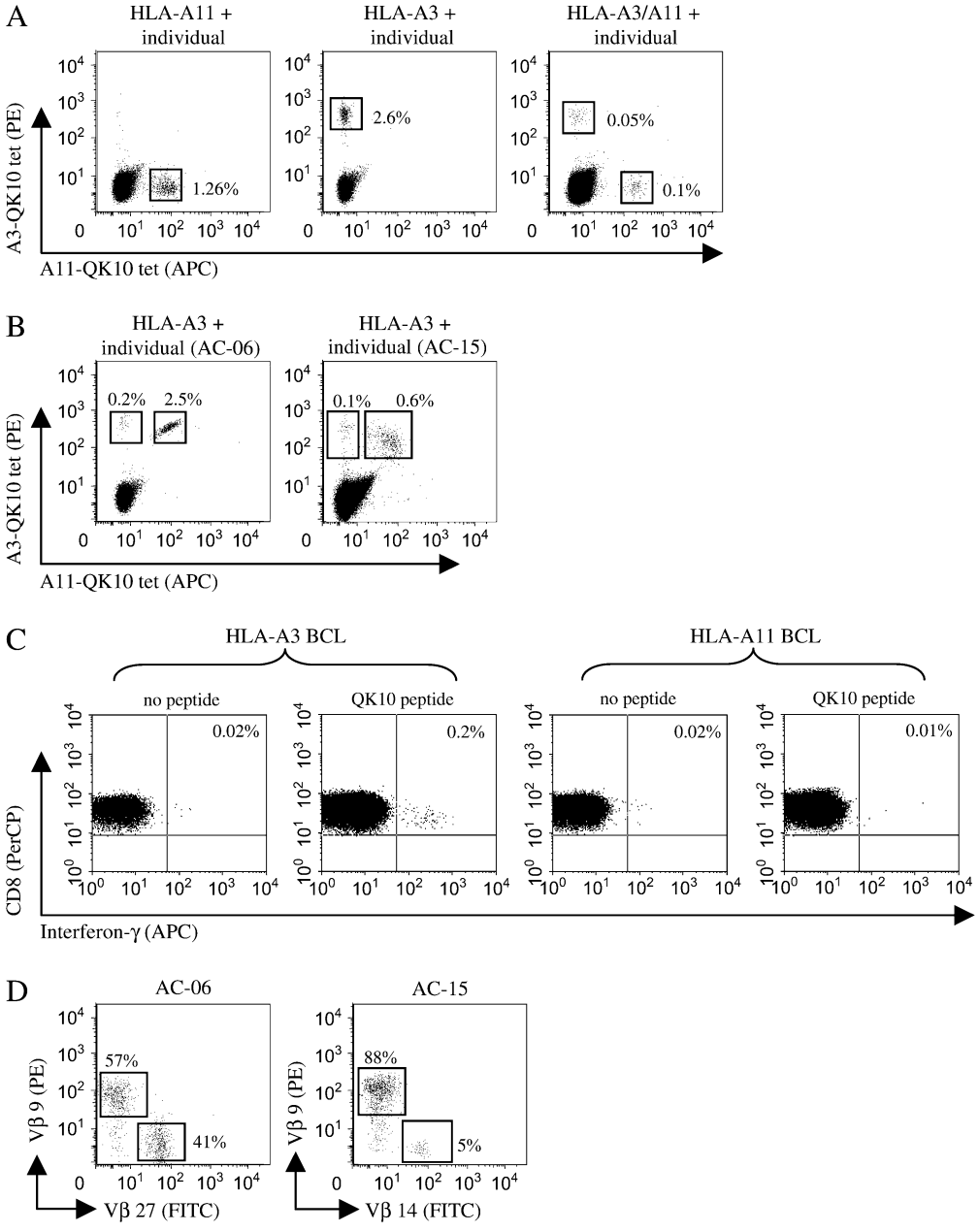

Fig. 1. Flow cytometric assessment of HLA-A3/-A11 cross-reactivity of QK10-specific CD8+ T cells. (A and B) Binding of CD8+ T cells to QK10/HLA-A3 or QK10/HLA-A11 tetramer in HIV-1-infected study persons expressing HLA-A3 only, HLA-A11 only or HLA-A3 and -A11. Cells were gated according to FSC/SSC characteristics and positivity for CD8. (C) IFN-γ secretion of CD8+ T cells from an HLA-A3-expressing study person following stimulation with QK10-loaded HLA-A3- (left panel) or -A11 (right panel) -restricted heterologous BCL. Percentages represent the proportion of CD8+ T cells secreting IFN-γ. (D) Dot plots indicating TCR Vβ chain usage of HLA-A3/-A11 cross-reactive QK10-specific CD8+ T cells. Cells were gated according to SSC/FSC characteristics, and positivity for CD8, HLA-A3/QK10 tetramer and HLA-A11/QK10 tetramer. Note the exclusive use of single Vβ chains on the surface of the cross-reactive cells.

their TCR clonotype pattern, we sorted these subsets of CD8+ T cells and analyzed the TCR α and β chain sequences, using a PCR amplification technique without bias for selected TCR Vα/β chains to ensure that epitope-specific clonotypes were represented in the PCR product with a relative frequency reflecting that in the originally sorted cell population. In contrast to the wide heterogeneity of clonotypes detected within some of the CD8+ T cell populations recognizing the QK10 peptide exclusively in the context of HLA-A3 or HLA-A11, we found that the A3/A11 cross-reactive QK10-specific CD8+ T cells in these two study individuals were extremely oligoclonal and used two (AC-06) or three (AC-15) distinct TCR β chains, while only one TCR α clonotype variant was detected in each of these two populations, both at the level of amino acid and nucleotide residues (Table 3). To further assess whether additional sub-dominant TCR α chain variants were used by these cross-reactive CD8+ T cell populations, we also used 21 different Vα chain-specific primers paired with Cα outer/inner primers for the assessment of the clonotypic α chain composition in these two different cell populations. This technique also failed to reveal additional TCR α chain variants, thus supporting our previous finding of only one TCR α chain being used in combination with two (AC-06) or three (AC-15) TCR β chain clonotypes in these HLA-A3/-A11 cross-reactive QK10-specific CD8+ T cell populations.

A similarly oligoclonal composition of TCR α and β chain clonotypes was detected in the QK10-specific CD8+ T cells exclusively restricted to HLA-A3 in these two study subjects (Table 3). Staining with TCR Vβ chain-specific antibodies confirmed the presence of distinct clonotypic populations within these cross-reactive QK10-specific CD8+ T cells and excluded the possibility of two different TCR β chain variants being expressed by the same lymphocyte (22) (Fig. 1D). Interestingly, the vast majority (85%) of the TCR β chain CDR3 regions of the cross-reactive QK10-specific CD8+ T cell populations contained the amino acid sequence motif ASS-R, which was not detectable in any β chain CDR3 region amplified from non-cross-reactive QK10-specific CD8+ T cells in these two study persons and four additional study individuals (two HLA-A3 expressing, one HLA-A11 expressing and one HLA-A3 and -A11 expressing; Table 4) with non-cross-reactive QK10-specific CD8+ T cells (Table 3). No specific amino acid

Table 3. Clonotypic composition of QK10-specific CD8+ T cells recognizing their target peptide in the context of both HLA-A3 and -A11 (A) or only in the context of HLA-A3 (B) or -A11 (C)

Patient	TCR β chain		TCR α chain	
A. TCRs recognizing the QK10 peptide presented by HLA-A3 and HLA-A11				
AC-06	Vβ27	ASSVRTGELF-J2.2 (16/31)	Vα29	AASFTQNGLT-J45 (26/26)
AC-06	Vβ9	ASSERDSQYQETQY-J2.5 (15/31)		
AC-15	Vβ9	ASSARAFPEGNQPQH-J1.5 (31/42)	Vα39	AVVAQGGSEKLV-J57 (26/26)
AC-15	Vβ7.9	ASSYSGQGAAGELF-J2.2 (5/42)		
AC-15	Vβ14	ASSPVLYEQY-J2.7 (6/42)		
B. TCRs recognizing the QK10 peptide presented only by HLA-A3				
AC-06	Vβ6.2	ASGEVGELF-J2.2 (42/46)	n.d.	
AC-06	Vβ27	ASRETGWGNQPQH-J1.5 (4/46)		
AC-15	Vβ10.2	ASSETNRVEMEAF-J1.1 (32/46)	Vα8.6	AVSDPGFKTI-J9 (27/27)
AC-15	Vβ24	ATSAGRQRDTGELF-J2.2 (9/46)		
AC-15	Vβ19	ASSKYNEQF-J2.1 (3/46)		
AC-15	Vβ7.9	ASSPPGQVGANVLT-J2.6 (2/46)		
AC-132	Vβ6.6	ASSYSRGSGNTIY-J1.3 (19/42)	Vα21	AVLTPLEGGKLI-J23 (3/20)
AC-132	Vβ6.6	ASSPYRGPNTEAF-J1.1 (1/42)	Vα3	GTENSGGYQKVT-J13 (2/20)
AC-132	Vβ10.3	AISAGASFVTRSTDTQY-2.1 (6/42)	Vα9.2	ALSAPSGGYQKVT-J13 (5/20)
AC-132	Vβ10.3	AIRSTDTQY-J2.1 (1/42)	Vα13.1	AADYFQKLV-J8 (6/20)
AC-132	Vβ6.1	ASRQQGFVFEAKNIQY-J2.4 (3/42)	Vα19	PSLLGGATNKLI-J32 (6/20)
AC-132	Vβ6.1	ASSEEVEAF J1.1 (1/42)		
AC-132	Vβ20.1	SAPTSGSAAF-J1.1 (3/42)		
AC-132	Vβ20.1	ASRDSIQFSSNQPQH-J1.5 (1/42)		
AC-132	Vβ6.2/3	ASSYSMTSGSFSDLGAKNIQY-J2.4 (1/42)		
AC-132	Vβ6.2/3	ASRPGPVKNTGELF-J2.2 (1/42)		
AC-132	Vβ9	ASSLYHNTGELF-J2.2 (1/42)		
AC-132	Vβ9	ASSGGAHFSKIPLAGYNEQF-J2.1 (1/42)		
AC-132	Vβ5.4	ASSRTDFTAGELF-J2.2 (1/42)		
AC-132	Vβ27	ASSLTGHPYEQY-J2.7 (1/42)		
AC-132	Vβ28	ASSPGEKYEQY-J2.1 (1/42)		
AC-88	Vβ20.1	SARGAGGFTHYEQY-J2.7 (21/21)	n.d.	
AC-117	Vβ7.9	ASSLVGFANTGELF-J2.2 (13/36)	n.d.	
AC-117	Vβ27	ASSSSGRGLGIQY-J2.4 (23/36)		
C. TCRs recognizing the QK10 peptide presented only by HLA-A11				
AC-88	Vβ19	ASSVSGFSTDTQY-J2.3 (9/14)	Vα1.2	AVDAGNNRKLI-J38 (15/15)
AC-88	Vβ29	SVLKVGTSGFNEQF-J2.1 (3/14)		
AC-88	Vβ29	SVPKVGTSGFNEQF-J2.1 (1/14)		
AC-88	Vβ29	SVPKTGTSGFNEQF-J2.1 (1/14)		
AC-174	Vβ29.1	SVVAGGPGDTQY-J2.3 (29/29)	n.d.	

Table 4. Clinical and demographical characteristics of the study patients

Patient I.D.	Sex	Race	Date of assessments (years post-presentation)	HLA class I types
AC-06	m	w	7 years	A3; B7; Cw7
AC-15	m	w	6.5 years	A1, 3; B7, 8; Cw7
AC-88	m	w	2.2 years	A3, A11; B27, 38; Cw2, 12
AC-117	m	w	2 years	A2, A3; B44; Cw5, 7
AC-132	m	w	<6 months	A3, A68; B14, 44; Cw8, 16
AC-174	m	w	1.5 years	A11, A68; B55, B78; Cw3, 16

sequence motif was detected in the CDR1, CDR2 or CDR3 region of the α chains of the HLA-A3/-A11 cross-reactive QK10-specific CD8+ T cells in comparison to non-HLA-A3/-A11 cross-reactive cells (Table 3). Taken together, these data indicate that HLA-A3/-A11 cross-recognition of QK10-specific CD8+ T cells can be mediated by single cross-reactive TCRs with a specific TCR α and β chain clonotype.

Antigenic avidity of HLA-A3/-A11 cross-reactive QK10-specific T cells

To determine if the observed cross-reactive TCRs had a different avidity for the recognition of the QK10 peptide when presented by either HLA-A3 or HLA-A11, we kinetically analyzed the off-rate of the HLA-A3/QK10 and the HLA-A11/QK10 tetramers from the corresponding TCRs, using a recently published protocol of tetramer dissociation assays (18). In study persons AC-15, we observed an almost identical decay rate of both tetramers, suggesting that the TCRs bind to structurally similar or identical regions within HLA-A3 or HLA-A11 (Fig. 2). In study person AC-06, the HLA-A3 tetramer dissociated moderately slower from the respective TCR compared with the HLA-A11 tetramer, indicating a slightly higher avidity of the TCR to the QK10 peptide in the context of HLA-A3 (Fig. 2). Finally, we assessed whether the TCRs cross-reactive with HLA-A3 and HLA-A11 also bind to the QK10 peptide in the context of other HLA-A3 like alleles, such as A31, A33 or A68, using intracellular IFN-γ staining following stimulation of the cross-reactive QK10-specific CD8+ T cells with heterologous HLA-A31-, A33- or A68-expressing BCLs pulsed with the target peptide. Yet, in both study persons, no antigen-specific IFN-γ secretion was observed following stimulation with these BCL, while clear populations of IFN-γ-secreting cells were observed after stimulation with QK10-pulsed HLA-A3- or -A11-expressing BCLs (Fig. 3). Overall, these data show that while the identified cross-reactive TCRs bind with almost equivalent avidity to the QK10 peptide when presented by HLA-A3 or HLA-A11, they do not appear to be able to recognize their cognate antigen in the context of the other members of the HLA-A3 superfamily.

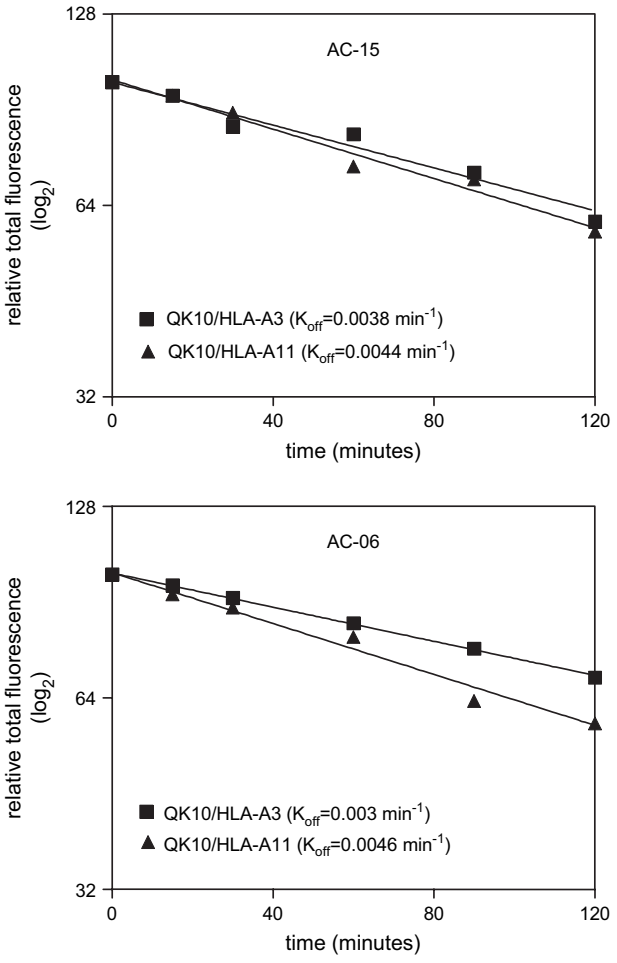

Fig. 2. TCR affinity of HLA-A3/-A11 cross-reactive QK10-specific CD8+ T cells. Plots indicate the dissociation of QK10/HLA-A3 and QK10/HLA-A11 tetramers from HLA-A3/-A11 cross-reactive TCRs.

Discussion

Due to shared peptide-binding motifs, HLA class I alleles from the same superfamily can present identical viral epitopes; however, it is unclear at present to what degree this promiscuous peptide presentation within one HLA superfamily is associated with promiscuous peptide recognition by cross-reactive TCRs. Here, we conducted a detailed analysis to determine the cross-presentation and cross-recognition of a panel of previously described optimal HLA-A3/-A11-restricted HIV-1 epitopes in HIV-1-infected individuals expressing either HLA-A3 or HLA-A11, the two most frequent members of the HLA-A3 superfamily. Our data show that the vast majority of these epitopes can be equally well recognized in HIV-1-infected individuals expressing HLA-A3 or -A11. Yet, using HLA-A3 and HLA-A11 tetramers refolded with the frequently recognized HIV-1 Nef QK10 peptide, we found that HLA-A3/-A11 cross-recognition of this epitope was highly infrequent during natural infection and, in fact, was only detected in two out of 25 study persons. In these two study persons, HLA-A3/-A11 cross-recognition of this epitopes was mediated by single cross-reactive TCRs. Thus, these data show that HLA-A3 and HLA-A11, despite very small differences in the amino acid sequence, exhibit clear immune distinctiveness and that promiscuous recognition of HIV-1 epitopes presented by HLA-A3 or HLA-A11 occurs only very rarely during natural infection.

In previous studies, a total of four different HIV-1-specific CD8+ T cell epitopes [AIFQSSMTK (RT, 158–166),

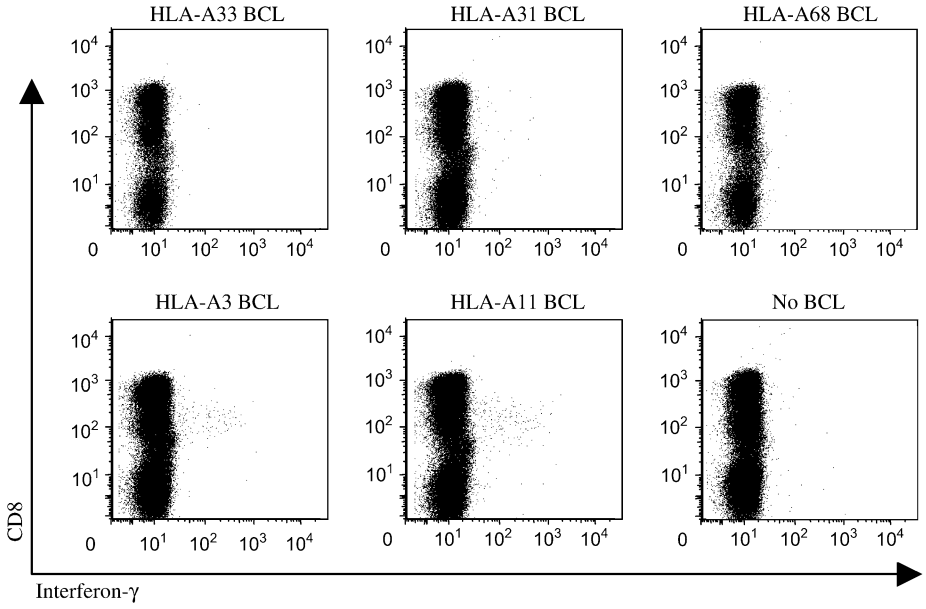

Fig. 3. HLA-A3/-A11 cross-reactive QK10-specific TCRs do not recognize QK10 in the context of other members of the HLA-A3 superfamily. Dot plots indicate IFN-γ secretion of CD8+ T cell following stimulation with HLA-A3, -A11, -A31, -A33 or -A68 expressing BCL in one study subject AC-15.

QVPLRPMTYK (Nef, 73–82), AVFIHNFKRK (Int, 179–188) and AVDLSHFLK (Nef, 84–92)] were defined that can be promiscuously recognized in individuals expressing either HLA-A3 or -A11 alleles (23). Here we extended this observation by identifying 13 additional optimal HIV-1 CD8+ T cell epitopes that can be recognized in carriers of either HLA-A3 or -A11 alleles. Importantly, at least in the case of HLA-A3, the prevalence of peptide recognition depended on the peptide binding affinity to the respective HLA molecule. Thus, these data show that specific HIV-1 epitopes, although they can principally be presented by HLA-A3 and HLA-A11, have differential degrees of immunogenicity and are associated with distinct recognition frequencies in the context of HLA-A3 or HLA-A11.

We subsequently attempted to determine if the promiscuous presentation of these peptides by members of the HLA-A3 superfamily is associated with HLA-A3/-A11 cross-reactive T cell recognition. Yet, at least in the context the most frequently targeted HIV-1 Nef QK10 peptide, HLA-A3/-A11 cross-recognition was extremely infrequent and occurred only in 8% of our study cohort. This observation contrasts to the study of Threlkeld *et al*. (24), who detected HLA-A3/-A11 cross-recognition of the HIV-1 RT epitope AK9 in two out of four HIV-1 individuals analyzed, thus suggesting that promiscuous epitope recognition among HLA class I alleles belonging to the HLA-3 superfamily is a fairly common event. The selection of naturally occurring CD8+ T cells with the capacity to cross-recognize antigens in the context of various members of the HLA-A3 superfamily was also suggested by other studies (25–29), although the frequency of these cross-reactive T cell populations remained undefined in these investigations.

In previous studies, cross-reactive HLA-A3/-A11 antigen-specific CD8+ T cells have been identified *in vitro* and *in vivo*, although in some of these investigations (25, 27, 29), the cross-recognition was apparently not mediated by cross-reactive TCRs but rather by multiple monospecific TCRs being present in a clonotypically heterogenous population of antigen-specific CD8+ T cells. In other studies, cross-recognition of identical peptides in the context of various HLA-A3 like molecules was observed in clonotypically homogenous cells (24, 26, 27); however, it was unclear in these investigations whether cross-recognition was mediated by a single TCR or by two different TCRs expressed on the same cell, which can occur in up to 30% of T lymphocytes (30). Instead of chromium release assays that had been used in some of these former studies, we therefore performed flow cytometric assays with distinctively labeled tetramers to analyze HLA-A3/-A11 cross-reactivity at the single cell level. Moreover, we sorted CD8+ T cells binding to HLA-A3 and -A11 tetramers refolded with the frequently recognized HIV-1 Nef QK10 peptide and subsequently analyzed the TCR α and β chain clonotypes in the cross-reactive cell population. Interestingly, in each of the cross-reactive cell populations, only one TCR α chain variant was detected, both at the level of amino acid and nucleotide residues, in conjunction with two or three different TCR β chain variants. To exclude the theoretical possibility that the simultaneous expression of these different TCR β chain variants on the cell surface of a single cell (22, 31) accounted for the observed cross-recognition, and not the cross-reactivity of the TCRs themselves, we stained the cross-reactive cells with fluorophore-labeled antibodies recognizing the respective TCR β chain variants, revealing separate and distinct populations of QK10-specific CD8+ T cells with individual TCR β chain usage. Thus, our data clearly indicate that the observed HLA-A3/-A11 cross-reactivity was mediated by single cross-reactive TCRs.

The detailed structural mechanisms that account for the HLA-A3/-A11 cross-reactivity of the TCRs described in this manuscript are unclear at present but will be important to explore in future studies. Based on the current understanding of TCR peptide-HLA class I interaction, it appears likely that these cross-reactive TCRs function either by focusing on features that are conformationally similar among HLA alleles belonging to the HLA-A3 superfamily or by adapting to conformationally dissimilar regions of the peptide-MHC class I complexes. In contrast, the described monospecific TCR recognizing the QK10 peptide exclusively in the context of HLA-A3 or -A11 are likely to bind to structures that are conformationally distinct between QK10–HLA-A3 and QK10–HLA-A11 complexes (10). Interestingly, the structural analysis of TCR-mediated antigen recognition indicated that most of the TCR–HLA class I interaction is achieved by the CDR1 and the CDR3 regions within the TCR α chain, while the direct interaction with the antigenic peptide appears to be executed by the CDR3 α and β chain regions (32, 33). Moreover, in the absence of crystal structure data on TCRs restricted to HLA-A3 like molecules, Li *et al.* (10, 34) have attempted to dock the structure of the human A6 TCR, restricted to HLA-A2 and specific for the Tax peptide (33), onto the structure of the HLA-A11–QK10 complex. Interestingly, this model revealed that strong interactions were formed between the α1 helix of HLA-A11 and the CDR1α and CDR2α loops of the TCR, while none of the three β chain CDR regions appeared to contact the HLA-A11 molecule. In addition, bulging residues P4 and P5 of the QK10 peptide formed numerous interactions with the CDR3α, 3β and 1α loops in this model. Overall, these results might correspond to our finding that in each of the two HLA-A3/-A11 cross-reactive CD8+ T cell populations detected, only one TCR α chain was detected in the context of two to three TCR β chain variants. It is therefore tempting to speculate that the HLA class I cross-reactivity of these TCRs was predominantly mediated by the binding specificities of the TCR α chains. However, we cannot entirely exclude that our TCR sequencing techniques failed to amplify additional TCR α chain clonotypes that are used by these cross-reactive T cell populations. The future analysis of the interaction of HLA-A3/-A11 cross-reactive TCRs with their target peptide–HLA complex might allow for the precise identification of structural features responsible for promiscuous antigen recognition. Moreover, the sequences of these cross-reactive TCRs might be relevant for the fabrication of broadly applicable soluble TCRs for diagnostic and therapeutic *ex vivo* and *in vivo* purposes (35) and for immunotherapy approaches against HIV-1 infection based on TCR gene transfer technology (36).

In summary, our data indicate that although HLA-A3 and HLA-A11 can present a large panel of different HIV-1-specific CD8+ T cell epitopes, the presentation of these epitopes is associated with specific and exclusive TCR recognition in the vast majority of cases. Promiscuous recognition of an HIV-1 epitope in the context of both HLA-A3 and HLA-A11, however, can be mediated by certain combinations of TCR α and β chain clonotypes. The three-dimensional analysis of the interaction of these cross-reactive TCRs with their target peptide-HLA complex might allow for the identification of structural components responsible for promiscuous antigen recognition.

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Abbreviations

APC	allophycoerythrin
BCLs	EBV-transformed B-lymphoblastoid cell lines
ELISPOT	enzyme-linked immunospot
NIH	National Institutes of Health
MGH	Massachusetts General Hospital
PE	R-phycoerythrin
RT	reverse transcription
SFC	spot forming cell
SMART	switching mechanism at 5′ end of RNA transcript

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