transmission rate, and HLA allele prevalence. Models would need to include factors such as the selection of compensatory mutations to slow reversion rates, and antiretroviral therapy access that would slow transmission rates.

HLA adaptation to certain $CD8^+$ T-cell responses may also alter currently established HLA associations with slow disease progression. Data here suggest that, whereas 25 years ago HLA-B*51 was protective in Japan[11,12], this is no longer the case (Supplementary Fig. 2). The apparent increase in I135X frequency in Japan over this time supports the notion that HLA-B*51 protection against HIV disease progression hinges on availability of the HLA-B*51-restricted TAFTIPSI response. However, whether this is the case remains unknown.

For HLA-B*27 and HLA-B*57, there is more clear-cut evidence that their association with HIV control depends on the Gag-specific epitopes presented and analysed here[4,7,13–15,18,19]. For each of the HLA-B*27- and HLA-B*57-associated Gag mutations studied, an *in vitro* fitness cost or *in vivo* reversion has been observed. A strong correlation between variant frequency and HLA prevalence even for rapidly reverting variants can be explained, either by mutant acquisition exceeding reversion rate (Fig. 4D), or by selection of compensatory mutations slowing or halting reversion altogether. The clearest example of the latter is the HLA-B*27-associated R264K mutation, 'corrected' by S173A[19]. Compensatory mutations are also well described for the HLA-B*57-associated Gag mutations[14,18]. These data suggest that the escape mutations in these HLA-B*27- and HLA-B*57-restricted epitopes are accumulating over time. Several studies have now demonstrated that transmission of viruses encoding escape mutants in the critical Gag epitopes to individuals expressing the relevant MHC class results in failure to control viraemia[2,21,22]. The accumulation at the population level of these escape mutations in HLA-B*27 and HLA-B*57 Gag epitopes is therefore likely to reduce the facility of these alleles to slow HIV disease progression.

The longer-term consequences of this process for immune control of HIV are unknown. Loss of currently immunodominant epitopes would promote subdominant $CD8^+$ T-cell responses, which can be more effective[23,24]. Also, the adapted virus provides new epitopes that can be presented, potentially with beneficial effects. In hepatitis C virus, for example, HLA-A*0301 holds a particular advantage, but only against the specific strain of virus responsible for the Irish outbreak[25]. In HIV, HLA-B*1801 is associated with high viraemia in C clade but not in B clade infection[10,11,26]; the opposite applies to HLA-B*5301.

Thus, the data presented here, showing evidence that the virus is adapting to $CD8^+$ T-cell responses, some of which may mediate the well-established associations (HLA-B*57, HLA-B*27 and HLA-B*51) with immune control of HIV, highlight the dynamic nature of the challenge for an HIV vaccine. Important questions to be addressed include the speed and extent of sequence change, particularly in Gag, the most effective target for $CD8^+$ T-cell responses[1,7,13,21]. The induction of broad Gag-specific $CD8^+$ T-cell responses may be a successful vaccine strategy, but such a vaccine will be most effective if tailored to the viral sequences prevailing, and thus may need to be modified periodically to keep pace with the evolving virus. Moreover, the strong associations between certain HLA class molecules, such as HLA-B*57, HLA-B*27 and HLA-B*51, and slow disease progression may decline as the epidemic continues, particularly where these HLA alleles are highly prevalent, and where HIV transmission rates are high.

METHODS SUMMARY

Overall 2,875 subjects were studied, from 9 previously established study cohorts. These cohorts comprised subjects from North America, the Caribbean, Europe, sub-Saharan Africa, Australasia and Asia. All subjects were antiretroviral-therapy-naive. Apart from the London acute cohort ($n = 142$), all cohorts comprised chronically infected subjects. The 14 variants studied are well-defined escape mutations within well-characterized $CD8^+$ T-cell epitopes, and included those persisting after transmission and likely to have little effect on viral fitness ($n = 5$), as well as those shown previously to reduce viral fitness ($n = 9$). Autologous HIV-1 sequences, and HLA class I types, were determined for all study subjects. The replicative capacity of I135X variants selected within the HLA-B*51-restricted epitope TAFTIPSI (RT 128–135) was assessed via *in vitro* competition assays and also via longitudinal follow-up of HLA-B*51-negative subjects infected acutely with I135X variants. Polymorphism frequency in the study cohorts was compared with prevalence of the relevant HLA molecule in the study cohort using a logistic regression model taking into account the different numbers of study subjects in each cohort. Demonstration of an HLA allele driving escape at Gag 146 in the Japanese cohort was undertaken first by identification of an association between HLA-B*4801 and A146P, subsequent definition of an HLA-B*4801-restricted $CD8^+$ T-cell response to a novel epitope Gag 138–147 (LI10), and finally demonstration that A146P reduced viral recognition by LI10-specific $CD8^+$ T cells.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Information Accession numbers for newly determined viral sequences are included in Supplementary Information. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to P.G. (philip.goulder@paediatrics.ox.ac.uk).

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Original article

Escape mutation selected by Gag28-36-specific cytotoxic T cells in HLA-A*2402-positive HIV-1-infected donors

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Abstract

Gag-specific CTLs are known to have stronger ability to control HIV-1 replication than others that are protein-specific. Therefore, the analysis of Gag escape mutants is expected to clarify the mechanisms of immune control in HIV-1-infected donors. However, only a limited number of Gag escape mutants have been identified so far. A previous study suggested the possibility that Gag28-3R (KW9-3R) is an escape mutant from HLA-A*2402-restricted KW9-specific CTLs but did not show any evidence of it. Here we sought to demonstrate that KW9-3R is selected as escape mutant by KW9-specific CTLs. KW9-specific CTLs showed a remarkable reduction in recognition of target cells infected with the KW9-3R mutant. The sequence analysis of HIV-1 from 58 HIV-1-infected individuals showed that the frequency of the KW9-3R mutant was significantly higher in HLA-A*2402$^+$ individuals than in HLA-A*2402$^-$ individuals. Longitudinal analysis of an HLA-A*2402$^+$ individual with HIV-1 early infection showed that this escape mutant was selected over an approximately 2-year period. These results together indicate that Gag28-3R is an escape mutant selected by HLA-A*2402-restricted KW9-specific CTLs. Further analysis of this epitope will clarify the role of HIV-1-specific CTLs in the control of HIV-1 among the Japanese population, since 70% of them carry this allele.

Keywords: HIV-1; Cytotoxic T-lymphocytes; Escape

1. Introduction

Human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocytes (CTLs) have an important role in the control of HIV-1 replication during acute and chronic phases of an HIV-1 infection [1–3]. However, CTLs cannot completely eradicate HIV-1, because HIV-1 can escape from the host immune system by various mechanisms, including mutations of its immunodominant CTL epitope. The substitution of just a single amino acid within a CTL epitope is crucial for the failure of binding to HLA class I molecules or of the interaction between the T cell receptors (TCRs) of specific CTLs and the peptide-HLA class I complex. Both mechanisms result in the loss of CTL activities against target cells infected with mutant HIV-1 and contribute to the selection of a virus that can escape from CTLs [4–10]. There are many studies demonstrating that CTL-mediated immune pressure selects CTL escape variants during both acute and chronic HIV-1 and simian immunodeficiency virus (SIV) infection [11,12] and that the appearance of the escape mutants could result in the loss of immune control and disease progression [4,13]. The escape of HIV-1 from CTL responses has been proposed to be an important obstacle for HIV-1 vaccine development [14,15].

Gag-specific CTLs are known to have a stronger ability to control HIV-1 replication than others that are protein-specific [16]. Therefore, it is assumed that the appearance of Gag escape mutant results in the failure of HIV-1 control by Gag-specific CTLs. In fact, several such cases have been reported [4,7,17,18]. Analysis

Abbreviations: HIV-1, human immunodeficiency virus type 1; CTL, cytotoxic T-lymphocytes; HLA, human leukocyte antigens.

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of Gag escape mutants is very important to clarify the mechanisms of AIDS pathogenesis and AIDS vaccine development.

Approximately 70% of the Japanese population expresses HLA-A*2402 [19]. Gag28-36 (KW9) is the only immunodominant epitope of 3 epitopes presented by this allele [20,21]. A previous study showed that Gag28-3R (KW9-3R) was detected in 2 of 3 Japanese HIV-1-infected HLA-A*2402$^+$ individuals and that HLA-A*2402-restricted KW9-specific CTLs did not recognize the KW9-3R peptide [22]. However, it was still unclear whether the appearance of the mutant was associated with HLA-A*2402 and if the HLA-A*2402-restricted KW9-specific CTLs selected the mutants.

Here, we sought to clarify that KW9-3R is an escape mutant from KW9-specific CTLs by demonstrating that specific CTLs showed markedly reduced killing activity towards target cells infected with KW9-3R mutant virus. In addition, we investigated whether this mutant is more highly detectable in HLA-A*2402$^+$ donors than in HLA-A*2402$^-$ donors in order to clarify whether this mutant is selected at the population level. Furthermore, we performed longitudinal analysis of this mutant in a case of early HIV-1-infection.

2. Materials and methods

2.1. Patient samples

Informed consent was obtained from all subjects according to the Declaration of Helsinki. For sequence analysis, blood specimens were collected in EDTA. Plasma and peripheral blood mononuclear cells (PBMCs) were separated from heparinized whole blood. Patient HLA type was determined by standard sequence-based genotyping.

2.2. Sequence of autologous virus

Viral RNA was extracted from samples of plasma from HIV-1-infected patients by the use of a QIAamp MinElute Virus spin kit (QIAGEN), and cDNA was synthesized from the RNA with SuperScript RNase H-Reverse Transcriptase and random primer (Invitrogen). The Gag region was amplified by nested PCR using Taq DNA polymerase (Promega). The PCR products were then agarose gel-purified and sequenced directly or cloned by use of a TOPO TA cloning kit (Invitrogen). All DNA sequencing was performed using a BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems) and an ABI PRISM 310 Genetic Analyzer.

2.3. Cells

C1R cells expressing HLA-A*2402 (C1R-A*2402) were previously generated [23] and were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 0.15 mg/ml hygromycin B. 721.221-CD4-A*2402 cells were generated by transfection of the CD4 gene and the HLA-A*2402 gene into 721.221 cells, and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 0.15 mg/ml hygromycin B.

2.4. Generation of CTL clones

Peptide-specific CTL clones were generated from an established peptide-specific bulk CTL culture by seeding 0.8 cells/well into U-bottomed 96-well microtiter plates (Nunc, Roskilde, Denmark) together with 200 μl of cloning mixture (RPMI 1640 medium supplemented with 10% FCS and 200 U/ml human recombinant interleukin-2, 5×10^5 irradiated allogeneic PBMC from a healthy donor, and 1×10^5 irradiated C1R-A*2402 cells prepulsed with a 1 μM concentration of the corresponding peptide, KW9 [KYKLKHIVW]). Wells positive for growth after about 2 weeks were transferred to 48-well plates together with 1 ml of the cloning mixture. The clones were examined for CTL activity by the standard ^{51}Cr release assay. All CTL clones were cultured in RPMI 1640–10% FCS supplemented with 200 U/ml recombinant human interleukin-2 and were stimulated weekly with irradiated target cells prepulsed with the appropriate HIV-1-derived peptide.

2.5. HIV-1 clones

NL-432gag^{SF2} was created by replacing the NL-432 gag gene with the SF2 gag gene. For NL-432gag^{SF2} KW9-3R, the mutation was introduced by site-directed mutagenesis (Invitrogen) based on overlap extension.

2.6. CTL assay for target cells pulsed with HIV-1 peptide

Cytotoxicity activity was measured by the standard ^{51}Cr release assay, as previously described [24]. Target cells (2×10^5) were incubated for 60 min with 100 μCi $Na_2^{51}CrO_4$ in saline and then washed 3 times with RPMI 1640 medium containing 10% newborn calf serum (NCS). Labeled target cells (2×10^3/well) were added to 96-well round-bottom microtiter plates (Nunc) along with the appropriate amount of the corresponding peptide. After a 1 h incubation, effector cells were added, and the mixtures were then incubated for 4 h at 37 °C. The supernatants were collected and analyzed with a gamma counter. Spontaneous ^{51}Cr release was determined by measuring the counts per minute (cpm) in supernatants from wells containing only target cells (cpm spn). Maximum ^{51}Cr release was determined by measuring the cpm in supernatants from wells containing target cells in the presence of 2.5% Triton X-100 (cpm max). Specific lysis was defined as (cpm exp − cpm spn)/(cpm max − cpm spn) × 100, where "cpm exp" is the counts per minute in the supernatant in the wells containing both the target and effector cells.

2.7. CTL assay for target cells infected with HIV-1

721.221-CD4-A*2402 cells were exposed to the virus for several days. The cells were used as target cells for a CTL assay when infection of approximately 80% cells was confirmed by intracellular staining for HIV-1 p24 antigen. Infected cells were labeled with ^{51}Cr as described above. Labeled target cells were added along with effector cells into

round-bottom microtiter plates (Nunc), and the mixtures were incubated for 6 h at 37 °C.

2.8. Intracellular cytokine (ICC) production assay

PBMCs from HLA-A*2402-positive HIV-1-infected patients were stimulated with KW9 peptide (1 μM) in culture medium (RPMI 1640 medium supplemented with 10% FCS and 200 U/ml recombinant human IL-2). After 14 days in culture, the cells were assessed for IFN-γ production activity by using a FACSCalibur. Briefly, bulk cultures were stimulated with C1R-A*2402 cells pulsed with KW9 peptide (1 μM) for 2 h at 37 °C. Brefeldin A (10 μg/ml) was then added, and the cultures were continued for an additional 4 h. Cells were collected and stained with phycoerythrin (PE)-labeled anti-CD8 monoclonal antibody (mAb; Dako Corporation, Glostrup, Denmark). After having been treated with 4% paraformaldehyde solution, the cells were permeabilized in permeabilization buffer (0.1% saponin and 20% NCS in phosphate-buffered saline) at 4 °C for 10 min and stained with fluorescein isothiocyanate (FITC)-labeled anti-IFN-γ mAb (PharMingen, San Diego, CA). After a thorough washing with the permeabilization buffer, the cells were analyzed by using the FACSCalibur.

2.9. HLA-peptide tetrameric complexes

The tetrameric complexes were synthesized as previously described [25]. Briefly, an ectodomain of HLA class I proteins and human β_2 microglobulin, produced in *Escherichia coli* that had been transformed with the relevant expression plasmids, were first solubilized in denaturing buffer containing 8 M urea and refolded in refolding buffer in the presence of a synthesized peptide for 48 h at 4 °C. The resultant 45-kDa complex was purified by size-exclusion (Superdex G75; Amersham Pharmacia Biotech UK, Ltd, Buckinghamshire, England) and anion-exchange (MonoQ column; Amersham Pharmacia Biotech UK, Ltd, Buckinghamshire, England) chromatographies. The purified complexes were enzymatically biotinylated at a BirA recognition sequence located at the C terminus of the heavy chain and were mixed with PE- or allophycocyanin (APC)-conjugated avidin (Molecular Probes) at a molar ratio of 4:1.

2.10. Tetramer analysis

Cryopreserved PBMC of HIV-1-infected donors were stained with PE- or APC-conjugated tetramers at 37 °C for 30 min. Following 2 washes with RPMI 1640–10% FCS, the cells were stained with FITC-conjugated anti-CD8 mAb at 4 °C for 30 min and subsequently analyzed using flow cytometry (FACSCalibur).

3. Results

3.1. Immunodominancy of HLA-A*2402-restricted Gag28-36 (KW9) epitope

A previous study using a cytolytic assay showed that KW9-specific T cells are detected in 4 of 12 chronically HIV-1-infected individuals [20]. We first re-evaluated whether KW9-specific $CD8^+$ T cells could be frequently detected in chronically HIV-1-infected Japanese donors carrying HLA-A*2402. PBMC from these donors and HIV-1-seronegative HLA-A*2402^+ donors as negative control were stimulated with the epitope peptides and cultured for 2 weeks. The specific $CD8^+$ T cells in these cultures were measured by performing an ICC production assay using KW9 epitope peptides (Fig. 1A). KW9-specific $CD8^+$ T cells were detected in 8 of the 12 individuals (Fig. 1B), confirming that KW9 is recognized as the immunodominant epitope in HIV-1-infected HLA-A*2402^+ Japanese donors.

3.2. Association of an HLA-A*2402 allele with KW9 mutations

To clarify whether KW9-specific CTLs select the escape mutants at the population level, we analyzed the sequences of the KW9 epitope and the flanking regions of this epitope of HIV-1 from 32 HLA-A*2402^+ and 26 HLA-A*2402^- donors. Several mutations were found, occurring at positions 1, 3, 7, and 8 in the KW9 epitope (Fig. 2). The frequency of the 3R mutation was significantly higher in the HLA-A*2402^+

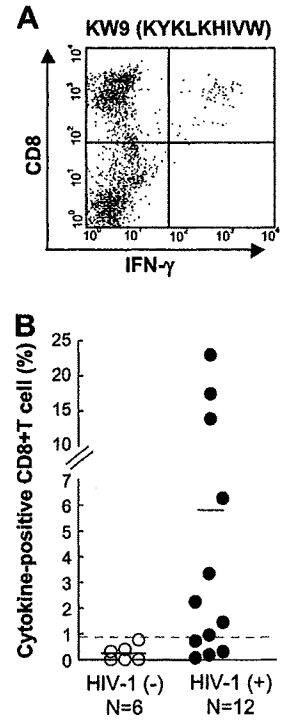


Fig. 1. HLA-A*2402-restricted KW9-specific $CD8^+$ T cells in chronically HIV-1-infected HLA-A*2402^+ individuals. (A) After PBMC from an HLA-A*2402^+ HIV-1-infected individuals had been stimulated with KW9 peptides for 2 weeks, KW9-specific $CD8^+$ T cells were detected by measuring IFN-γ-producing $CD8^+$ T cells in the culture stimulated with KW9 peptide. A representative result is shown in "A". (B) Summary of ICC assays for HLA-A*2402^+ HIV-1-infected and HIV-1-uninfected individuals. The average + 2 SD of IFN-γ-producing $CD8^+$ T cells in HIV-1-uninfected individuals was defined as a positive value (>0.87%). Dotted bars indicate the average + 2 SD; and horizon bars, the average in HIV-1-infected individuals.

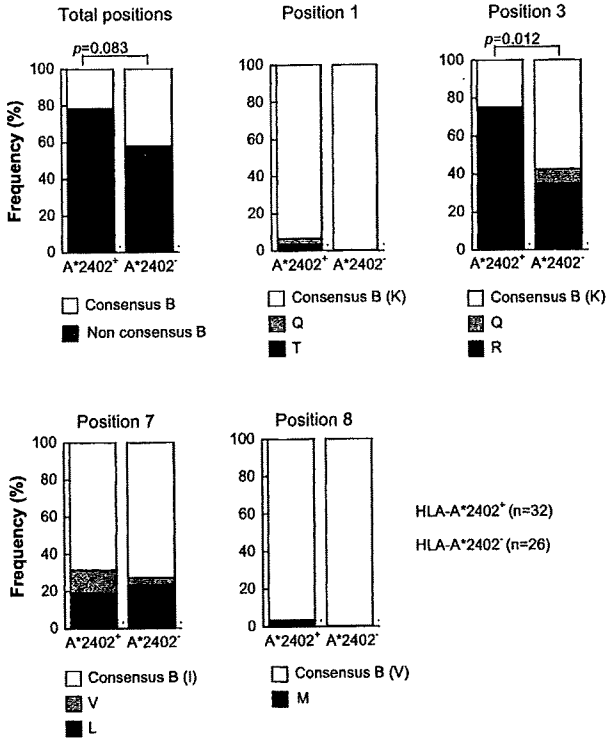


Fig. 2. Frequency of mutations in KW9 epitopes. The KW9 epitope sequence was analyzed in HLA-A*2402-positive and HLA-A*2402-negative individuals chronically infected with HIV-1. The consensus sequence of this epitope in clade B is KYKLKHIVW. The frequency of mutations in the total sequence of this epitope and those at a given position of the epitope are shown for both HLA-A*2402-positive and HLA-A*2402-negative donors. The *P* values were determined by Fisher's exact test.

donors than in the HLA-A*2402$^-$ donors ($P = 0.012$, Fig. 2). In the flanking region, there was no significant difference between HLA-A*2402$^+$ and HLA-A*2402$^-$ individuals (data not shown). These results together suggest that only the 3R mutation was selected by the KW9-specific CTLs.

3.3. 3R is an escape mutant from KW9-specific CTLs

To clarify that 3R is an escape mutant from KW9-specific CTLs, we investigated whether or not the KW9-specific CTLs could recognize the KW9-3R mutant epitope. We first tested the activity of KW9-specific CTL clones to kill target cells prepulsed with the KW9-3R mutant peptide. Two KW9-specific CTL clones and KW9-specific bulk CTLs effectively killed target cells prepulsed with the KW9 wild-type peptide but showed reduced ability to kill those prepulsed with the KW9-3R mutant peptide (Fig. 3A,C). Furthermore, to analyze whether or not KW9-specific CTLs could kill target cells infected with the KW9-3R mutant virus, we generated NL-432gag^{SF2} virus in which the NL-432 gag gene was replaced with the SF2 gag gene as well as NL-432gag^{SF2} carrying the KW9-3R epitope (NL-432gag^{SF2} KW9-3R). The KW9-specific CTL clones and KW9-specific bulk CTLs effectively killed the target cells infected with NL-432gag^{SF2}, whereas they failed to kill those infected with NL-432gag^{SF2} KW9-3R

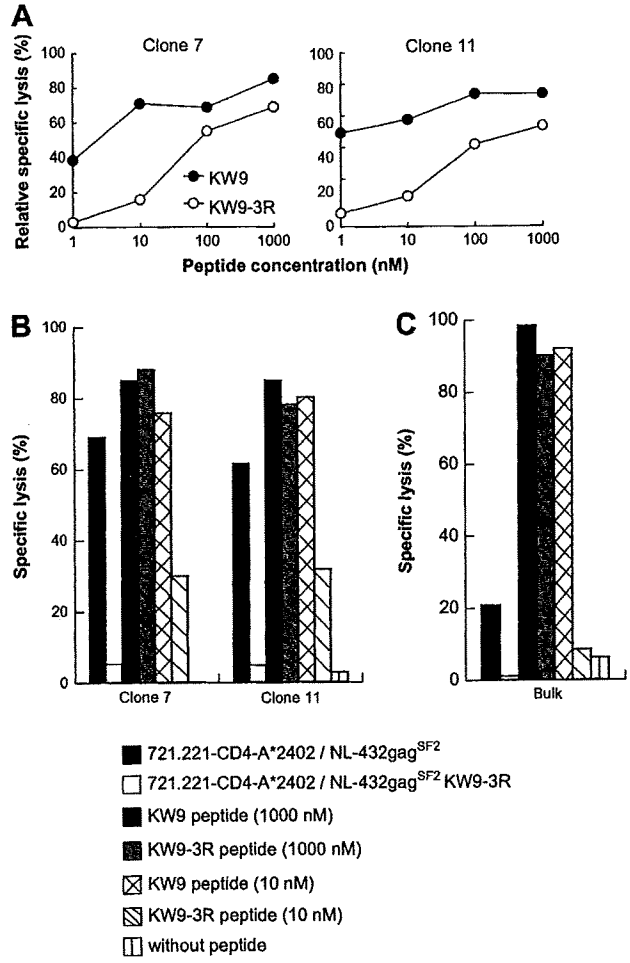


Fig. 3. Cytotoxic activity of KW9-specific CTLs against target cells pulsed with KW9-3R peptide and infected with NL-432gag^{SF2} KW9-3R. (A) Cytolytic activities of KW9-specific CTL clones to kill C1R-A*2402 cells pulsed with KW9 or KW9-3R peptide. C1R-A*2402 cells were prepulsed with various concentrations of KW9 or KW9-3R peptide. Cytolytic activities of KW9-specific CTL clones were measured at an effector-to-target ratio of 2:1. (B) Cytotoxic activity of KW9-specific CTL clones against HIV-1-infected 721.221-CD4-A*2402. 721.221-CD4-A*2402 cells were infected with NL-432gag^{SF2} or NL-432gag^{SF2} KW9-3R. NL-432gag^{SF2}-infected (80.5% of total cells were p24 antigen-positive) or NL-432gag^{SF2} KW9-3R-infected (81.9% of total cells were p24 antigen-positive) 721.221-CD4-A*2402 were used as target cells at an E:T ratio of 2:1. (C) Cytotoxic activity of KW9-specific bulk CTL cells against HIV-1-infected or epitope peptide-pulsed 721.221-CD4-A*2402. NL-432gag^{SF2}-infected (45.4% of total cells were p24 antigen-positive), NL-432gag^{SF2} KW9-3R-infected (43.8% of total cells were p24 antigen-positive) 721.221-CD4-A*2402 or peptide-pulsed 721.221-CD4-A*2402 were used as target cells at an E:T ratio of 10:1.

(Fig. 3B,C), indicating that the Gag28-3R mutant is the escape one from KW9-specific CTLs.

*3.4. Longitudinal analysis of KW9 epitope and KW9-specific CD8$^+$ T cells in an HIV-1-infected HLA-A*2402$^+$ individual*

We furthermore performed longitudinal analysis of KW9-specific CTL responses and the KW9 epitope in an

HLA-A*2402$^+$ individual, KI-092, having an early HIV-1 infection. To investigate the number of Gag28-specific CTLs, we stained PBMCs from KI-092, who had been receiving STI therapy during the early phase, with the KW9-tetramer and anti-CD8 mAb. In this patient, KW9-specific CD8$^+$ T cells were effectively elicited during the early phase of the infection (Fig. 4A); however, the number of these cells became rapidly reduced within 10 months and remained low thereafter for approximately 4 years (Fig. 4B). We next analyzed the sequence of this epitope during the clinical course in this patient. As expected, the wild-type (WT) sequence of KW9 was found in the early phase. However, the patient showed the presence of both WT and 3R mutant viruses approximately 2 years later, and had only the 3R virus about 3 years later (Fig. 4C). These results strongly support the idea that KW9 is selected by KW9-specific T cells.

4. Discussion

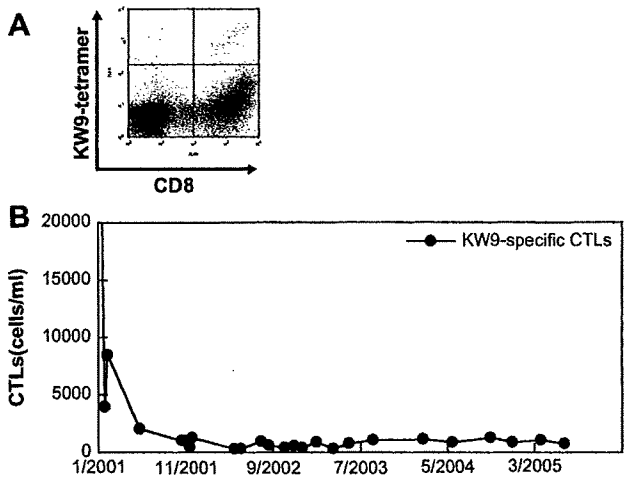
Our previous studies identified 3 HLA-A*2402-restricted Gag epitopes and showed that KW9-specific cytolytic activities were detected in only 4 of 12 chronically HIV-1-infected HLA-A*2402$^+$ donors but that other Gag epitope-specific cytolytic activities were hardly detected [20,21]. The present study using the ICC assay demonstrated that KW9-specific CD8$^+$ T cells were detected in 8 of the 12 chronically HIV-1-infected HLA-A*2402$^+$ donors tested. The difference in frequency of KW9-specific T cells between the 2 studies may have been due to the difference in the assays used. The present study confirmed that KW9 is the immunodominant epitope and showed that KW9-specific T cells are elicited in chronically HIV-1-infected HLA-A*2402$^+$ donors more than was previously speculated.

A previous study showed that the KW9-3R mutant was detected in 2 of 3 HIV-1-infected HLA-A*2402-positive Japanese individuals and that HLA-A*2402-resricted KW9-specific CTLs failed to kill the target cells pulsed with KW9-3R peptide [22]. That study implied only the possibility that KW9-3R is a mutation for escape from the specific CTLs. In the present study, we showed that KW9-specific CTLs effectively killed target cells infected with the WT virus but failed to kill those infected with the KW9-3R mutant virus. In addition, the sequence analysis of this epitope in 58 chronically HIV-1-infected individuals showed that the frequency of the KW9-3R mutation was significantly higher in HLA-A*2402$^+$ Japanese individuals than in the HLA-A*2402$^-$ individuals, indicating that this mutation was selected in an HLA-A*2402-restricted fashion at the population level. This finding also supports the idea that KW9-3R is an escape mutant. Approximately 40% of the HLA-A*2402$^-$ Japanese individuals were infected with the HIV-KW9-3R mutant, suggesting that this mutant has accumulated in the Japanese population and implying that this mutant does not revert in HLA-A*2402$^-$ hosts.

Seven of 8 HLA-A*2402$^+$ donors who induced KW9-specific CTLs shown in Fig. 1B had the 3R mutation, while all 4 HLA-A*2402$^+$ donors who did not induce KW9-specific CTLs had this mutation (data not shown). This result indicates that HLA-A*2402$^+$ donors who have the 3R mutation can still maintain KW9-specific memory or effector memory T cells. However, it still remains unclear that the 3R mutation elicits the 3R-specific T cells.

Escape mutations occur at some site within the CTL epitope, where the substitution of an amino acid abrogates HLA binding, reduces the recognition of the TCR, and/or interferes with efficient antigen processing. HLA-A*2402-binding peptides have 2 anchor residues, Tyr at position 2 and Phe, Leu, Ile or Trp at the carboxyl terminus [26,27]. Since position 3 is not an anchor residue and the substitution from Lys to Arg is speculated not to affect the binding affinity, the KW9-3R peptide may bind to HLA-A*2402 molecules with similar affinity to the WT peptide. Therefore, the escape mechanism of the KW9-3R mutant virus may involve the failure of the recognition of the TCR and/or the disruption of cellular processing of the KW9-3R peptide.

Longitudinal analysis of the KW9 epitope in an HLA-A*2402$^+$ individual having an early HIV-1 infection revealed that half of the HIV-1 isolates were the 3R mutant approximately 2 years after the early phase of the infection when only the wild-type HIV-1 was detected. These results suggest that this escape mutant is slowly selected by the specific T cells,



C

Sample date (M/D/Y)	KW9 epitope KYKLKHIVW	Number of clones
01/22/2001	---------	12/12
12/10/2002	---------	5/10
	--R------	4/10
	--R--R---	1/10
08/14/2003	--R------	7/7

Fig. 4. Longitudinal analysis of KW9-specific CD8$^+$ T cells and KW9 epitope in an HLA-A*2402$^+$ individual having an early HIV-1 infection. *Ex vivo* analysis of KW9-specific CTLs. KW9-specific CTLs in PBMC (2/1/2001) derived from an HLA-A*2402$^+$ individual, KI-092, who received STI therapy during the early phase of infection, were measured by using KW9-tetramer and anti-CD8 mAb (A). Results of *ex vivo* longitudinal analysis of KW9-specific CTLs (B) and the sequence of the KW9 epitope (C) derived from KI-092 are also shown.

and also support the idea that the 3R mutant is selected by KW9-specific CTLs. The analysis using the tetramers showed that the frequency of KW9-specific CTLs was reduced within 9 months. This finding implies that the 3R mutant had already appeared at this point in time.

The results obtained in the present study reveal that KW9-3R is selected as an escape mutant by KW9-specific CTLs. As this escape mutation has accumulated in the Japanese population, HLA-A*2402^{+} new hosts may become infected with the 3R mutant virus; and the CTLs of such patients may fail to respond to this mutant virus. Further studies of this escape mutant will be necessary to clarify HIV-1-specific CTL responses in Japanese and other Asian populations.

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Strong Ability of Nef-Specific CD4$^+$ Cytotoxic T Cells To Suppress Human Immunodeficiency Virus Type 1 (HIV-1) Replication in HIV-1-Infected CD4$^+$ T Cells and Macrophages$^\nabla$

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A restricted number of studies have shown that human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic CD4$^+$ T cells are present in HIV-1-infected individuals. However, the roles of this type of CD4$^+$ T cell in the immune responses against an HIV-1 infection remain unclear. In this study, we identified novel Nef epitope-specific HLA-DRB1*0803-restricted cytotoxic CD4$^+$ T cells. The CD4$^+$ T-cell clones specific for Nef187-203 showed strong gamma interferon production after having been stimulated with autologous B-lymphoblastoid cells infected with recombinant vaccinia virus expressing Nef or pulsed with heat-inactivated virus particles, indicating the presentation of the epitope antigen through both exogenous and endogenous major histocompatibility complex class II processing pathways. Nef187-203-specific CD4$^+$ T-cell clones exhibited strong cytotoxic activity against both HIV-1-infected macrophages and CD4$^+$ T cells from an HLA-DRB1*0803$^+$ donor. In addition, these Nef-specific cytotoxic CD4$^+$ T-cell clones exhibited strong ability to suppress HIV-1 replication in both macrophages and CD4$^+$ T cells in vitro. Nef187-203-specific cytotoxic CD4$^+$ T cells were detected in cultures of peptide-stimulated peripheral blood mononuclear cells (PBMCs) and in ex vivo PBMCs from 40% and 20% of DRB1*0803$^+$ donors, respectively. These results suggest that HIV-1-specific CD4$^+$ T cells may directly control HIV-1 infection in vivo by suppressing virus replication in HIV-1 natural host cells.

Human immunodeficiency virus (HIV)-specific CD8$^+$ cytotoxic T cells (CTLs) play a central role in the control of HIV type 1 (HIV-1) during acute and chronic phases of an HIV-1 infection (5, 29, 34). However, HIV-1 escapes from the immune surveillance of CD8$^+$ CTLs by mechanisms such as mutations of immunodominant CTL epitopes and downregulation of major histocompatibility complex class I (MHC-I) molecules on the infected cells (9, 11, 12, 49). Therefore, most HIV-1-infected patients without highly active antiretroviral therapy (HAART) develop AIDS eventually.

HIV-1-specific CD4$^+$ T cells also play an important role in host immune responses against HIV-1 infections. An inverse association of CD4$^+$ T-cell responses with viral load in chronically HIV-1-infected patients was documented in a series of earlier studies (8, 36, 39, 41, 48), although the causal relationship between them still remains unclear (23). Classically, CD4$^+$ T cells help the expansion of CD8$^+$ CTLs by producing growth factors such as interleukin-2 (IL-2) or by their CD40 ligand interaction with antigen-processing cells and CD8$^+$ CTLs. In addition, CD4$^+$ T cells provide activation of macrophages, which can professionally maintain CD8$^+$ T-cell memory (17). On the other hand, the direct ability of virus-specific cytotoxic CD4$^+$ T cells (CD4$^+$ CTLs) to kill target cells has been widely observed in human virus infections such as those by human cytomegalovirus, Epstein-Barr virus (EBV), hepatitis B virus, Dengue virus, and HIV-1 (2, 4, 10, 19, 30, 31, 38, 50). Furthermore, one study showed that mouse CD4$^+$ T cells specific for lymphocytic choriomeningitis virus have cytotoxic activity in vivo (25). These results, taken together, indicate that a subset of effector CD4$^+$ T cells develops cytolytic activity in response to virus infections.

HIV-1-specific CD4$^+$ CTLs were found to be prevalent in HIV-1 infections, as Gag-specific cytotoxic CD4$^+$ T cells were detected directly ex vivo among peripheral blood mononuclear cells (PBMCs) from an HIV-1-infected long-term nonprogressor (31). Other studies showed that up to 50% of the CD4$^+$ T cells in some HIV-1-infected donors can exhibit a clear cytolytic potential, in contrast to the fact that healthy individuals display few of these cells (3, 4). These studies indicate the real existence of CD4$^+$ CTLs in HIV-1 infections.

The roles of CD4$^+$ CTLs in the control of an HIV-1 infection have not been widely explored. It is known that Gag-specific CD4$^+$ CTLs can suppress HIV-1 replication in a human T-cell leukemia virus type 1-immortalized CD4$^+$ T-cell line (31). However, the functions of CD4$^+$ T cells specific for other HIV-1 antigens remain unclear. On the other hand, the abilities of CD4$^+$ CTLs to suppress HIV-1 replication in infected macrophages and CD4$^+$ T cells may be different, as in the case of CD8$^+$ CTLs for HIV-1-infected macrophages (17). In this study, we identified Nef-specific CD4$^+$ T cells and investigated their ability to kill HIV-1 R5 virus-infected macrophages and HIV-1 X4 virus-infected CD4$^+$ T cells and to suppress HIV-1 replication in the infected macrophages and

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CD4+ T cells. The results obtained in the present study show for the first time the ability of HIV-1-specific CD4+ CTLs to suppress HIV-1 replication in natural host cells, i.e., macrophages and CD4+ T cells.

MATERIALS AND METHODS

Patients. Informed consent was obtained from all subjects, in accordance with the Declaration of Helsinki. Plasma and PBMCs were separated from heparinized whole blood. The patients were sampled at the AIDS Clinical Center, International Medical Center of Japan, and the HLA types of the patients were determined by standard sequence-based genotyping. Patients with active opportunistic infections or psychological disorders and those treated with immunomodulatory agents were excluded.

Synthetic peptides. Peptides (17-mer) derived from the consensus sequence of the Nef protein of HIV-1 clade B were synthesized. These 17-mer peptides overlapped each other by 11 residues. For the feasibility of screening for T-cell epitopes, eight peptides were pooled in a cocktail. Peptides were prepared by using an automated multiple peptide synthesizer. The purity of the synthesized peptides was examined by mass spectrometry, and the peptides with >90% purity were used in the present study.

Cell surface and intracellular cytokine staining. For detection of intracellular cytokines of CD4+ T cells, PBMCs or Nef-specific CD4+ T-cell clones (effector cells) bulk cultured with peptides were stimulated with autologous EBV-transformed B-lymphoblastoid cells (B-LCLs) prepulsed with Nef-derived peptides or peptide cocktails (10^{-6} M) at an effector-to-stimulator (E/S) ratio of 1:4. The pulsed stimulator cells were washed twice in RPMI 1640–10% fetal calf serum (FCS) before use. The mixed cells were incubated for 6 h at 37°C in 5% CO_2. Brefeldin A (Sigma-Aldrich) was added at a concentration of 10 μg/ml after the first 2 h of incubation to inhibit secretion of cytokines. In order to determine the MHC-II restriction of the CD4+ T-cell epitopes, we also employed peptide-pulsed allogeneic B-LCLs with the HLA-DR allele partially matched or mismatched as stimulators in some assays. After a 6-hour incubation, the cells were stained with phycoerythrin (PE)-conjugated anti-human CD4 monoclonal antibody (MAb) (BD Biosciences, San Jose, CA). Then the cells were fixed, made permeable, stained with fluorescein isothiocyanate (FITC)-conjugated anti-human gamma interferon (IFN-γ) MAb (BD Biosciences, San Jose, CA), and analyzed by flow cytometry as previously described (16).

In order to determine the expression of cytotoxic effector molecules, we directly stained PBMCs or Nef-specific CD4+ T-cell clones with allophycocyanin (APC)-conjugated anti-human CD4 or PE-conjugated anti-human CD4 MAb (BD Biosciences, San Jose, CA) without any stimulation of the cells. Then the cells were fixed, made permeable, stained with FITC-conjugated anti-human perforin, PE-conjugated anti-human granzyme A, or Alexa 647-conjugated anti-human granzyme B MAb (BD Biosciences, San Jose, CA), and analyzed by flow cytometry as previously described (44).

To detect the degranulation of Nef-specific CD4+ T cells following antigen stimulation directly ex vivo, we incubated PBMCs with PE-conjugated anti-human CD107a MAb or PE-conjugated isotype control MAb in RPMI 1640–10% FCS containing the corresponding peptide (10^{-6} M), as previously described by Casazza et al. (10). Negative controls containing the PBMCs from the same individual but without peptides were also prepared. Cells were incubated for 6 h at 37°C in 5% CO_2. Brefeldin A was added at a concentration of 10 μg/ml after the first 2 h of incubation. Then, the cells were stained with APC-conjugated anti-human CD4 MAb and FITC-conjugated anti-human IFN-γ MAb and analyzed as described above.

Generation of Nef-specific CD4+ T-cell clones. Peptide-specific CD4+ T-cell clones were generated from an established peptide-specific bulk CD4+ T-cell culture by limiting dilution in U-bottom 96-well microtiter plates (Nunc, Roskilde, Denmark) together with 200 μl of cloning mixture (RPMI 1640 medium supplemented with 10% human serum from healthy donors and 200 U/ml recombinant human IL-2, 5×10^4 irradiated allogeneic PBMCs from a healthy donor as feeders, and 1×10^5 irradiated autologous EBV-transformed B-LCLs prepulsed with a 10^{-6} M concentration of the corresponding peptide). Wells positive for growth after 2 to 3 weeks were transferred to 48-well plates together with 1 ml of the cloning mixture. The clones were examined for specific IFN-γ-producing ability by intracellular cytokine staining. All CD4+ T-cell clones were cultured in RPMI 1640–10% human serum from healthy donors supplemented with 200 U of recombinant human IL-2/ml and were stimulated weekly with irradiated autologous B-LCLs prepulsed with the appropriate epitope peptide.

Blocking of CD4+ T-cell responses. To determine the MHC-II restriction of Nef-specific CD4+ T-cell responses, we blocked the T-cell receptor–MHC-II interaction by using human MHC-II molecule-specific MAbs L243 (anti-HLA-DR), B7/21 (anti-HLA-DP), and Hu-11 and Hu-18 (anti-HLA-DQ4+5+6 and anti-HLA-DQ7+8+9, respectively), which were kindly donated by Y. Nishimura. Autologous B-LCLs prepulsed with the Nef epitope were incubated with the appropriate antibody (10 μg/ml) for 1 h on ice. Subsequently, the cells were washed in RPMI 1640–10% FCS and then incubated with Nef-specific CD4+ T-cell clones (effector cells) at an E/S ratio of 1:2 for 6 h. Brefeldin A was added to the cultures (10 μg/ml) 4 h prior to termination of the cultures. To evaluate the ability of the effector cells to produce IFN-γ under blocking conditions, we stained the cells after stimulation with PE-conjugated anti-human CD4 MAb. Then the cells were fixed, made permeable, and stained with FITC-conjugated anti-human IFN-γ, as described above.

Intracellular cytokine production (ICC) assays for stimulator cells infected with recombinant vaccinia virus. Autologous B-LCLs were infected with 10 PFU per cell of recombinant vaccinia virus expressing HIV-1 Nef (rVac-Nef) or wild-type vaccinia virus (Vac-WT) and cultured for 16 h at 37°C in 5% CO_2. The infected cells were washed twice with RPMI 1640–10% FCS and then incubated with Nef-specific CD4+ T-cell clones (effector cells) at an E/S ratio of 1:4 for 6 h. Brefeldin A was present in the cultures (10 μg/ml) for the last 4 h. To evaluate the ability of the effector cells to produce IFN-γ, we stained the cells with PE-conjugated anti-human CD4 MAb after stimulation. Then the cells were fixed, made permeable, and stained with FITC-conjugated anti-human IFN-γ, as described above.

ICC assays for stimulator cells pulsed with heat-inactivated HIV-1 particles. The virus particles of HIV-1 NL-432 and its Nef-defective mutant were generated by the HIV-1 clones and were heat inactivated at 56°C for 30 min. Autologous B-LCLs were incubated with the inactivated virus particles at 0.5 μg/ml (p24 antigen concentration) for 16 h at 37°C in 5% CO_2. The pulsed cells were washed twice with RPMI 1640–10% FCS and then incubated with Nef-specific CD4+ T-cell clones (effector cells) at an E/S ratio of 1:4 for 6 h. Brefeldin A was present in the cultures (10 μg/ml) for the last 4 h. To evaluate the ability of effector cells to produce IFN-γ after stimulation, we sequentially stained the cells with PE-conjugated anti-human CD4 MAb, fixed them, made them permeable, and then stained them with FITC-conjugated anti-human IFN-γ MAb, as described above.

ICC assay for stimulator cells transfected with Nef-GFP fusion mRNA. For stimulator cells endogenously expressing Nef-green fluorescent protein (GFP) fusion proteins, m7GpppG-capped and poly(A)-tailed Nef-GFP fusion mRNA or GFP mRNA was delivered to autologous B-LCLs by electroporation, as previously described (46). Briefly, B-LCLs were suspended in a serum-free medium (Opti-MEM; Invitrogen Life Technologies) at the cell density of 2×10^6 cells/ml, mixed with 10 μg of mRNA, and electroporated by using a Gene Pulser device (Bio-Rad). The cells were immediately transferred to RPMI 1640–10% FCS, incubated at 37°C for 1.5 to 3 h, and then mixed with Nef-specific CD4+ T-cell clones (effector cells) at an E/S ratio of 1:4. B-LCLs transfected with GFP mRNA were prepared as negative controls. Flow cytometry revealed that more than 60% of the viable B-LCLs expressed GFP. The cell mixtures were incubated for 6 h, and brefeldin A (10 μg/ml) was present for the last 4 h of the incubation. To evaluate the ability of the effector cells to produce IFN-γ after stimulation, we performed surface and intracellular cytokine staining to the cells, as described above.

Isolation and culture of macrophages and CD4+ T cells. Monocytes and CD4+ T cells were isolated from PBMCs of an HLA-DRB1*0803-positive or HLA-DRB1*0403-positive healthy donor by using anti-human CD14 MAb-coated and anti-human CD4 MAb-coated magnetic beads (magnetically activated cell sorting beads; Miltenyi Biotec, Bergisch Gladbach, Germany), respectively. The isolated monocytes were cultured in complete medium containing macrophage colony-stimulating factor (50 ng/ml) for 1 week before use. The isolated CD4+ T cells were cultured for 1 week in complete medium containing IL-2 (200 U/ml) and IL-4 (2.5 ng/ml) and stimulated with OKT3 anti-CD3 MAb (10 μg/ml) every 3 days during the culture period. These cultured macrophages and CD4+ T cells were infected with HIV-1 as previously described (17, 45).

HIV-1 clones. Infectious proviral clones of an X4 HIV-1, pNL-432, and its Nef-defective mutant, pNL-Xh, which has a frameshift at a XhoI site (44th amino acid of the Nef protein), were kindly donated by Y. Koyanagi (Kyoto University, Kyoto, Japan). The infectious proviral clone of $pJRFL_{NL\text{-}432Nef}$ was previously constructed by exchanging the Nef region of R5 strain JRFL with that of NL-432 (17).

CTL assay. The cytotoxicity of Nef-specific CD4+ T-cell clones against B-LCLs or HIV-1-infected target cells was measured by a standard ^{51}Cr release assay as previously described (17). Briefly, target cells (2×10^5) were incubated for 60 min with 100 μCi of $Na_2{}^{51}CrO_4$ in saline and washed three times with RPMI 1640 medium containing 10% NCS. Labeled target cells (2×10^3/well)

were seeded in a 96-well round-bottom microtiter plate (Nunc). For the assays of B-LCLs, the desired amount of the corresponding peptide was coincubated with labeled target cells for 1 h. Then, effector cells were added at various E/T ratios, and the mixtures were incubated for 4 h at 37°C. The supernatants were collected and analyzed with a gamma counter. The spontaneous ^{51}Cr release was determined by measuring the cpm in the supernatant in the wells containing only target cells (cpm spn). Maximum release was determined by measuring the release of ^{51}Cr from the target cells in the presence of 2.5% Triton X-100 (cpm max). Specific lysis was calculated by using the formula (cpm exp − cpm spn)/(cpm max − cpm spn) × 100 (%), where cpm exp is the counts per minute in the supernatant in the wells containing both target and effector cells.

Suppression of HIV-1 replication by HIV-1-specific CTLs. The ability of HIV-1 Nef-specific $CD4^+$ CTLs to suppress HIV-1 replication was examined as previously described (45). Briefly, macrophages or $CD4^+$ T cells were incubated with a given HIV-1 clone for 6 h at 37°C in 5% CO_2. After two washes with RPMI 1640–10% FCS, the cells were cocultured with the $CD4^+$ CTL clones. From days 3 to 9 after infection, 10 μl of culture supernatant was collected, and the concentration of p24 antigen was measured by use of an enzyme immunoassay (HIV-1 p24 antigen enzyme-linked immunosorbent assay kit (ZeptMetrix, Buffalo, NY). The percentage of suppression of HIV-1 replication was calculated as follows: % suppression = (1 − concentration of p24 Ag in the supernatant of HIV-1-infected cells cultured with HIV-1-specific CTLs/concentration of p24 Ag in the supernatant of HIV-1-infected cells culture without the CTLs) × 100.

RESULTS

Identification and characterization of two HIV-1 Nef-specific $CD4^+$ T-cell epitopes. PBMC from two HIV-1-seropositive individuals, KI-010 and KI-197, were cultured for 14 days after stimulation with either of four peptide cocktails comprising eight 17-mer overlapping Nef peptides. Specific IFN-γ production by each PBMC culture was tested by using intracellular IFN-γ staining after restimulating the cells with autologous EBV-transformed B-LCLs prepulsed with the corresponding peptide cocktail. Cocktail 1 and cocktail 4 induced specific IFN-γ-producing $CD4^+$ T cells among the PBMCs from KI-010 and KI-197, respectively (Fig. 1A). In order to determine which peptide was responsible for the specific $CD4^+$ T-cell responses in the peptide cocktails, we subsequently stimulated the responding PBMC cultures with autologous B-LCLs pulsed with each peptide included in the corresponding peptide cocktails. Nef17-7 and Nef17-8 peptides induced specific $CD4^+$ T-cell responses by the PBMCs cultured from KI-010, whereas Nef17-31 and Nef17-32 peptides induced specific ones by those from KI-197 (Fig. 1B). Considering that the flanking residues also contribute a small part to the overall binding energy of MHC-II-binding peptides, the core binding region is usually not the optimal ligand for MHC-II molecules. Therefore, we used the full-length 17-mer peptides Nef37-53 (Nef17-7) and Nef187-203 (Nef17-32) to generate $CD4^+$ T-cell clones for further studies. The clones specific for Nef37-53 and Nef187-203 epitopes were generated from KI-010 and KI-197, respectively.

In order to determine the HLA class II restriction molecules of these two $CD4^+$ T-cell epitopes, we employed HLA-DR-, -DP-, and -DQ-specific MAbs to block the T-cell receptor-HLA class II interaction between Nef-specific $CD4^+$ T cells and the stimulator cells. HLA-DR-specific MAb L243 blocked the recognition by both Nef37-53- and Nef187-203-specific $CD4^+$ T-cell clones after stimulation with the peptide-pulsed autologous B-LCLs, whereas HLA-DQ-specific MAb Hu11 or Hu18 and HLA-DP-specific MAb B7/21 failed to block it (Fig. 1C). These results indicate that these two epitope-specific T-cell responses were restricted by HLA-DR. To determine the

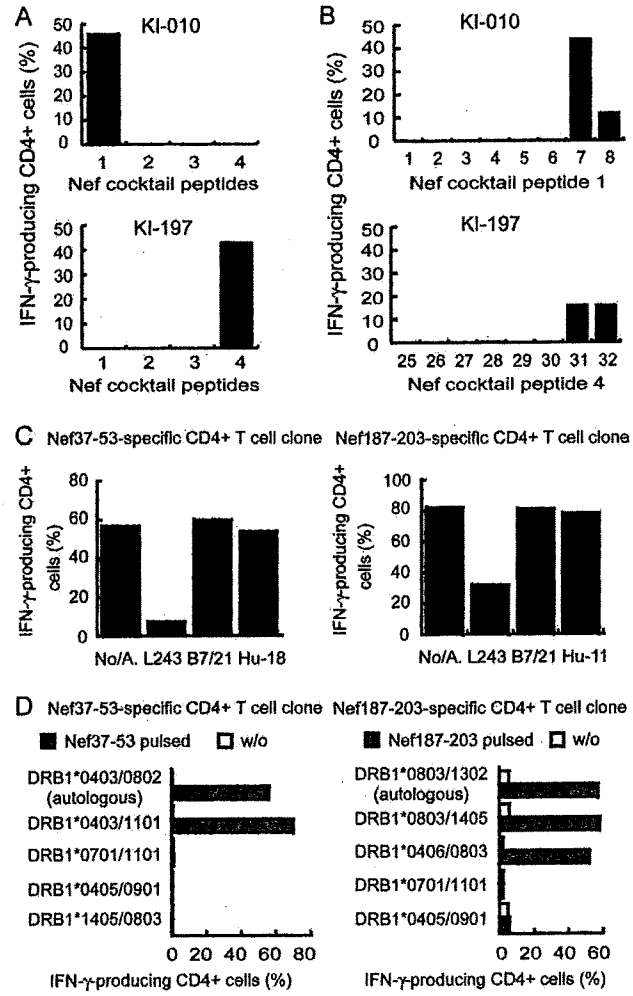

FIG. 1. Identification and characterization of two HIV-1 Nef-specific $CD4^+$ T-cell epitopes. (A) Induction of Nef-specific $CD4^+$ T cells from PBMCs of HIV-1-infected individuals. PBMCs from two HIV-1-seropositive donors (KI-010 and KI-197) were stimulated with cocktails comprising eight 17-mer overlapping Nef peptides and then were cultured for 2 weeks. IFN-γ-producing $CD4^+$ T cells (%) among these bulk-cultured PBMCs were detected by intracellular staining for IFN-γ after restimulation with autologous B-LCLs pulsed with the same cocktails. (B) IFN-γ-producing $CD4^+$ T cells induced by Nef single peptides. The PBMC bulk cultures that responded to the peptide cocktails were subsequently stimulated with B-LCLs pulsed with individual peptides included in those cocktails. IFN-γ-producing $CD4^+$ T cells (%) induced by single peptides were detected by intracellular staining for IFN-γ. (C) IFN-γ responses of Nef37-53-specific and Nef187-203-specific $CD4^+$ T-cell clones to the stimulation with peptide-pulsed B-LCLs were blocked by HLA-DR-specific antibody. Autologous B-LCLs prepulsed with epitope peptides were incubated with MHC-II-specific antibodies (No/A., no antibody; L243, anti-HLA-DR; B7/21, anti-HLA-DP; Hu11 and Hu18, anti-HLA-DQ) for 1 h. Then the two Nef epitope-specific $CD4^+$ T-cell clones were stimulated with the MHC-II-specific antibody-treated B-LCLs at an E/S ratio of 1:2. The percentage of IFN-γ-producing cells in the Nef-specific $CD4^+$ T-cell clones after stimulation was determined by intracellular staining for IFN-γ. (D) IFN-γ responses of Nef37-53-specific and Nef187-203-specific $CD4^+$ T-cell clones after stimulation with peptide-pulsed autologous B-LCL or peptide-pulsed allogeneic B-LCLs with partially matched and mismatched HLA-DR. The percentage of IFN-γ-producing cells among the Nef-specific $CD4^+$ T-cell clones after stimulation was determined by intracellular staining for IFN-γ.

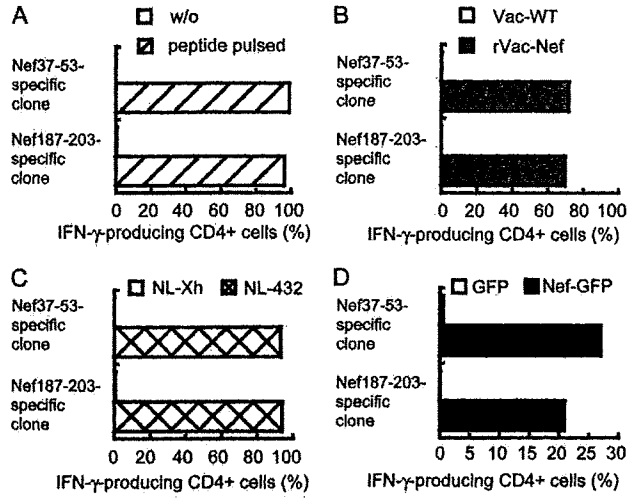

FIG. 2. Naturally occurring presentation of CD4+ T-cell epitopes. Nef37-53-specific and Nef187-203-specific CD4+ T-cell clones were stimulated with peptide-pulsed, recombinant vaccinia virus-infected, HIV-1 particle protein-pulsed, or Nef-GFP fusion mRNA-transfected autologous B-LCLs. The percentage of IFN-γ-producing cells among the Nef-specific CD4+ T-cell clones after stimulation was determined by intracellular staining for IFN-γ. (A) Nef-specific CD4+ T-cell clones were tested for their IFN-γ production after stimulation with B-LCLs prepulsed with appropriate epitope peptides (peptide pulsed) or those without peptides (w/o). (B) Nef-specific CD4+ T-cell clones were tested for IFN-γ production after stimulation with B-LCLs infected with rVac-Nef or Vac-WT. (C) Nef-specific CD4+ T-cell clones were tested for their IFN-γ production after stimulation with B-LCLs prepulsed with heat-inactivated HIV-1 particles of X4 strain NL-432 (NL-432) or those of its Nef-defective mutant, NL-Xh (NL-Xh). (D) Nef-specific CD4+ T-cell clones were tested for their IFN-γ production after stimulation with Nef-GFP fusion mRNA-transfected B-LCLs (Nef-GFP) or GFP mRNA-transfected B-LCLs (GFP). Approximately 60% of the stimulator cells were Nef+ or GFP+ cells.

exact restriction alleles, we stimulated Nef37-53-specific and Nef187-203-specific CD4+ T-cell clones with peptide-prepulsed B-LCLs from allodonors with partially matched or mismatched HLA-DR. The Nef37-53-specific CD4+ T-cell clone produced IFN-γ after stimulation with the corresponding peptide-pulsed B-LCLs from the donors sharing DRB1*0403, while the Nef187-203-specific clone produced IFN-γ after stimulation with the corresponding peptide-pulsed B-LCLs from the donors sharing DRB1*0803 (Fig. 1D). These results strongly suggest that the restriction alleles of CD4+ T-cell epitopes Nef37-53 and Nef187-203 were HLA-DRB1*0403 and HLA-DRB1*0803, respectively.

Naturally occurring presentation of CD4+ T-cell epitopes in rVac-Nef-infected or HIV-1 Nef protein-pulsed cells. To clarify the naturally occurring presentation of these two Nef epitopes, we investigated the ability Nef37-53-specific and Nef187-203-specific CD4+ T-cell clones to produce IFN-γ after stimulation of them with autologous B-LCLs infected with rVac-Nef or those pulsed with heat-inactivated virus particles. The Nef37-53-specific and Nef187-203-specific clones used in this assay showed similar abilities to produce IFN-γ (>95%) after the stimulation with peptide-pulsed autologous B-LCLs (Fig. 2A). The B-LCLs infected with rVac-Nef induced about 70% of the two Nef-specific CD4+ T-cell clones to produce IFN-γ, whereas those cells infected with Vac-WT did not induce any IFN-γ production (Fig. 2B). In addition, the B-LCLs pulsed with NL-432 virus particles induced more than 90% of the CD4+ T cells from the Nef-specific clones to produce IFN-γ, whereas those cells pulsed with NL-Xh (Nef-depleted) virus particles failed to induce IFN-γ production (Fig. 2C). This result suggests that the Nef-specific CD4+ T cells also recognized the epitope antigen presented through endogenous MHC-II processing pathways. However, it still remains possible that Nef proteins from cells expressing Nef killed by vaccinia virus or HIV infection were presented by the exogenous HLA class II pathway. To exclude this possibility, we used stimulator cells transfected with Nef-GFP mRNA. Nef-GFP mRNA-transfected autologous B-LCLs induced IFN-γ production from both Nef37-53-specific and Nef187-203-specific CD4+ T-cell clones, whereas GFP mRNA-transfected cells did not (Fig. 2D). In this assay, B-LCLs were used as stimulator cells within 3 h after the transfection. The frequency of dead cells among the Nef+ cells was approximately 0.6%. These results support the idea that endogenous HIV-1 Nef can be processed to MHC-II molecules in a manner similar to that of the previously observed endogenous presentation of HCMV CD4+ CTL epitopes (20). Thus, our results indicate that the Nef-specific CD4+ T cells recognized the epitope antigen presented through both exogenous and endogenous MHC-II processing pathways.

Cytotoxic activity and cytotoxic effector molecule expression of HIV-1 Nef-specific CD4+ T cells. Although antigen-specific CD4+ T cells are classically thought to function as helper T cells in antiviral immunity, HIV-1 Gag-specific cytotoxic CD4+ T cells were previously reported to exist (30–32, 50). In our study, Nef37-53-specific and Nef187-203-specific CD4+ T-cell clones were tested for their ability to lyse autologous B-LCLs incubated with the epitope peptide (1,000 nM) at an E/T ratio of 5:1 (Fig. 3A). The Nef187-203-specific CD4+ T-cell clone showed a strong lytic activity against autologous B-LCLs incubated with the peptide, whereas the Nef37-53-specific CD4+ T-cell clone did not lyse autologous B-LCLs pulsed with the peptide. Furthermore, we stained for three cytotoxic effector molecules in these Nef-specific CD4+ T-cell clones and found that the expression levels of perforin and granzyme B were much higher in the Nef187-203-specific clone than in the Nef37-53-specific one, whereas the two clones showed similar levels of granzyme A expression (Fig. 3B). Considering that Th clones have been shown to develop cytotoxic activity after long-term culture in vitro (15), we sought to detect the cytotoxic activity of these two Nef epitope-specific CD4+ T cells ex vivo. We employed flow cytometric analysis to measure the cell surface mobilization of CD107a (6, 14), because only a very small number of these epitope-specific CD4+ T cells are suspected to exist among the PBMCs of these patients; thus, these cells would fail to kill the target cells in a chromium release assay. Epitope-specific CD4+ T cells among the PBMCs from two HIV-1-seropositive donors, KI-010 and KI-197, could be detected at very low frequency by revealing their specific IFN-γ responses following peptide stimulation for 6 h (Fig. 3C). We then gated the IFN-γ-producing CD4+ T cells and compared the levels of cell surface expression of CD107 for these two types of CD4+ T cells. The results showed that about 50% of Nef187-203-specific CD4+ T cells expressed CD107a on their

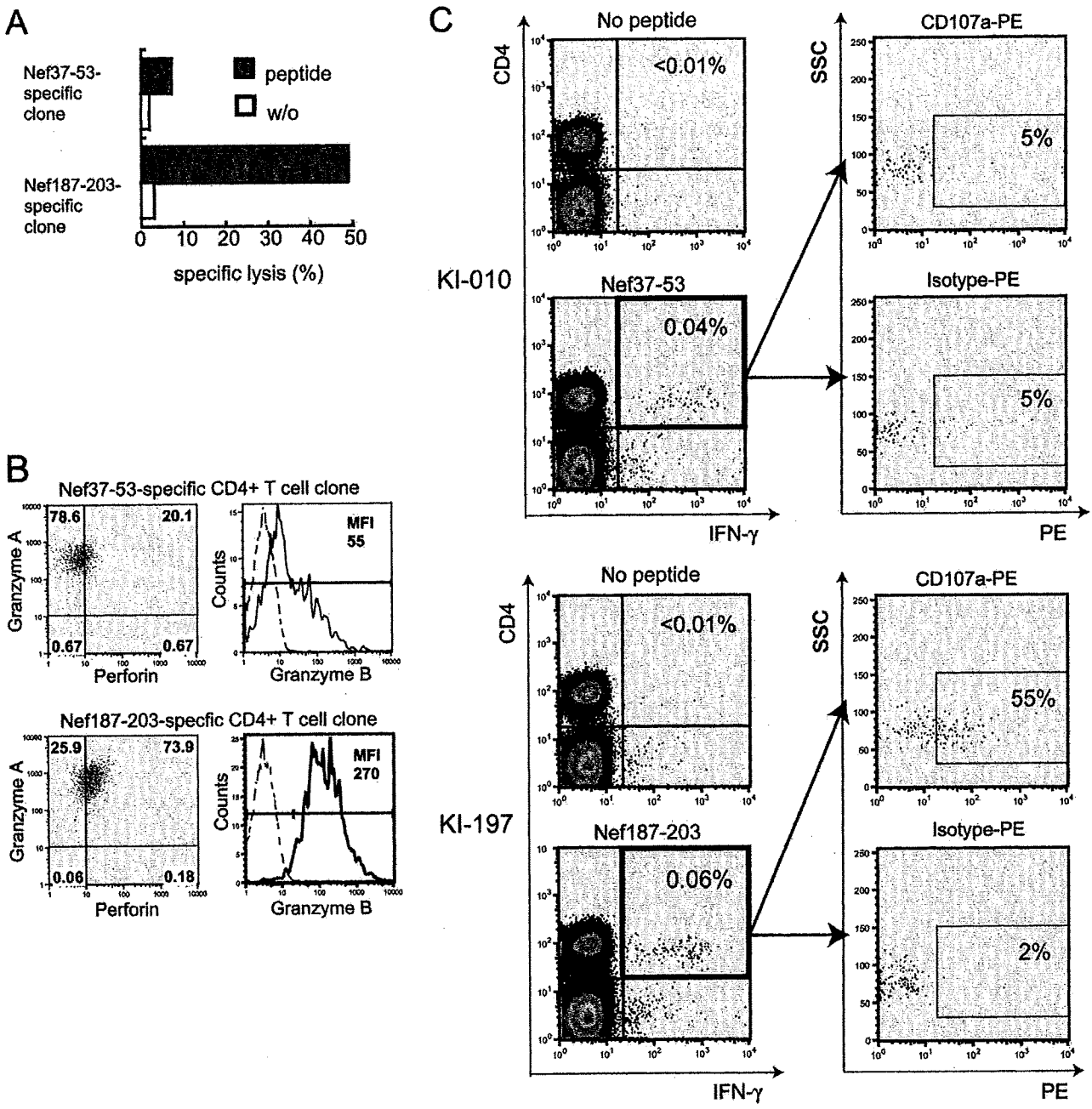

FIG. 3. Cytotoxic activity and cytotoxic effector molecule expression of HIV-1 Nef-specific $CD4^+$ T cells. (A) Cytotoxic activities of a KI-010-derived Nef37-53-specific $CD4^+$ T-cell clone and a KI-197-derived Nef187-203-specific $CD4^+$ T-cell clone against autologous B-LCLs incubated with the epitope peptides (1,000 nM) were measured by a standard ^{51}Cr release assay at an effector-to-target ratio of 5:1. Peptide, with peptide; w/o, without peptide. (B) Surface staining for CD4 and intracellular staining for perforin, granzyme A, and granzyme B were carried out on the Nef37-53-specific and Nef187-203-specific $CD4^+$ T-cell clones. The clones were stained without any stimulation. The stained clones were analyzed by flow cytometry, and the $CD4^+$ cells were gated. The expression levels of perforin and granzyme A are shown in dot plots. Values in dot plots show the frequencies (%) of the subsets among the $CD4^+$ T-cell clones. The expression levels of granzyme B are shown in histograms. Solid lines show the clones stained with anti-human granzyme B MAb; dashed lines show the same clones stained with isotype control antibody. Values in histograms show mean fluorescence intensities (MFI) of the solid lines. (C) Ex vivo analysis of CD107a surface expression on Nef37-53-specific and Nef187-203-specific $CD4^+$ T cells. PBMCs from two HIV-1-seropositive donors, KI-010 and KI-197, were incubated with or without their corresponding epitope peptide for 6 h. Then these PBMCs were stained with anti-CD4, anti-IFN-γ, and anti-CD107a or with mouse immunoglobulin G (IgG) MAb as an isotype control. Values in the IFN-γ/CD4 dot plots indicate the frequencies of IFN-γ-producing $CD4^+$ cells. The $CD4^+$ IFN-γ^+ cells in each PBMC population were gated, and then they were analyzed for the surface expression of CD107a. Values in the PE/side scatter (SSC) dot plots indicate the frequencies of the high-fluorescence subsets in the gated $CD4^+$ IFN-γ^+ population of the PBMCs stained with PE-conjugated anti-CD107a (CD107a-PE) and of the same PBMCs stained with PE-conjugated mouse IgG isotype MAb (Isotype-PE).

cell surfaces, whereas Nef37-53-specific CD4+ T cells did not, thus indicating that Nef187-203-specific CD4+ CTLs, but not Nef37-53-specific CD4+ T cells, have the ability to function as cytotoxic T cells.

Lysis of HIV-1-infected macrophages and CD4+ T cells by Nef187-203-specific cytotoxic CD4+ T cells. To investigate if the Nef-specific CD4+ T cells were able to kill HIV-1-infected target cells, we measured their cytotoxic activity against HIV-1-infected macrophages and CD4+ T cells. To exclude the possibility that different Nef sequences between two HIV-1 strains, NL-432 and JRFL, would affect the recognition of Nef-specific CD4+ CTLs, we used $JRFL_{NL\text{-}432Nef}$, a chimera R5 virus, with the Nef protein derived from the NL-432 strain in this study. Macrophages and CD4+ T cells from an HLA-DRB1*0803-positive healthy donor were infected with HIV-1 R5 strain $JRFL_{NL\text{-}432Nef}$ and X4 strain NL-432, respectively. Intracellular p24 staining of these cells showed that more than 80% of the cultured macrophages and CD4+ T cells were p24 antigen positive at day 3 postinfection, indicating the establishment of an HIV-1 infection in the cultured cells (Fig. 4A). Three Nef187-203-specific CD4+ CTL clones were used in our assays. They exhibited strong specific lysis of autologous B-LCLs incubated with 1,000 nM peptide; this lysis was dramatically decreased when the B-LCLs were incubated with 100 nM peptide (Fig. 4B), thus showing a lower sensitivity of peptide-pulsed target cells to Nef-specific CD4+ CTL clones than that of Nef-specific CD8+ CTL clones reported in our previous studies (18, 46). These Nef-specific CD4+ CTL clones killed both HIV-1-infected macrophages and CD4+ T cells, even at a decreased E/T ratio of 2:1 (Fig. 4C). The specific lysis of infected macrophages was higher than that of the infected CD4+ T cells. This difference may result from the intracellular p24 antigen expression levels of these two targets used in this assay (Fig. 4A).

Ability of HIV-1 Nef-specific cytotoxic CD4+ T cells to suppress HIV-1 replication in macrophages and CD4+ T cells. A previous study showed that Gag-specific CD4+ CTLs can suppress HIV-1 replication in human T-cell leukemia virus type 1-immortalized CD4+ T-cell line MT-2 (31). To clarify if CD4+ CTLs could also efficiently suppress HIV-1 replication in its natural host cells in vivo, we measured the ability of Nef-specific CD4+ CTLs to suppress the replication of HIV-1 in HIV-1-infected macrophages and CD4+ T cells in vitro. Macrophages and CD4+ T cells from an HLA-DRB1*0803-positive healthy donor were isolated, cultured, and then infected with HIV-1 $JRFL_{NL\text{-}432Nef}$ and NL-432 in vitro, respectively. To investigate the suppression ability of CD4+ CTLs by using an enzyme immunoassay, we measured p24 antigens in the supernatant of cultured HIV-1-infected target cells with or without a Nef187-203-specific CD4+ CTL clone at an E/T ratio of 0.1:1 (Fig. 5A). Two Nef187-203-specific clones revealed a strong ability to suppress HIV-1 replication in both HIV-1-infected macrophages and CD4+ T cells. The suppression ability of these T cell clones was E/T ratio dependent for both HIV-1-infected macrophages and CD4+ T cells (Fig. 5B), whereas the addition of an HLA class II-mismatched Nef37-53-specific CD4+ T-cell clone to HIV-1-infected macrophages or CD4+ T cells did not cause any suppression of p24 production (data not shown). Complete suppression of p24 production in both HIV-1-infected macrophages and CD4+ T cells

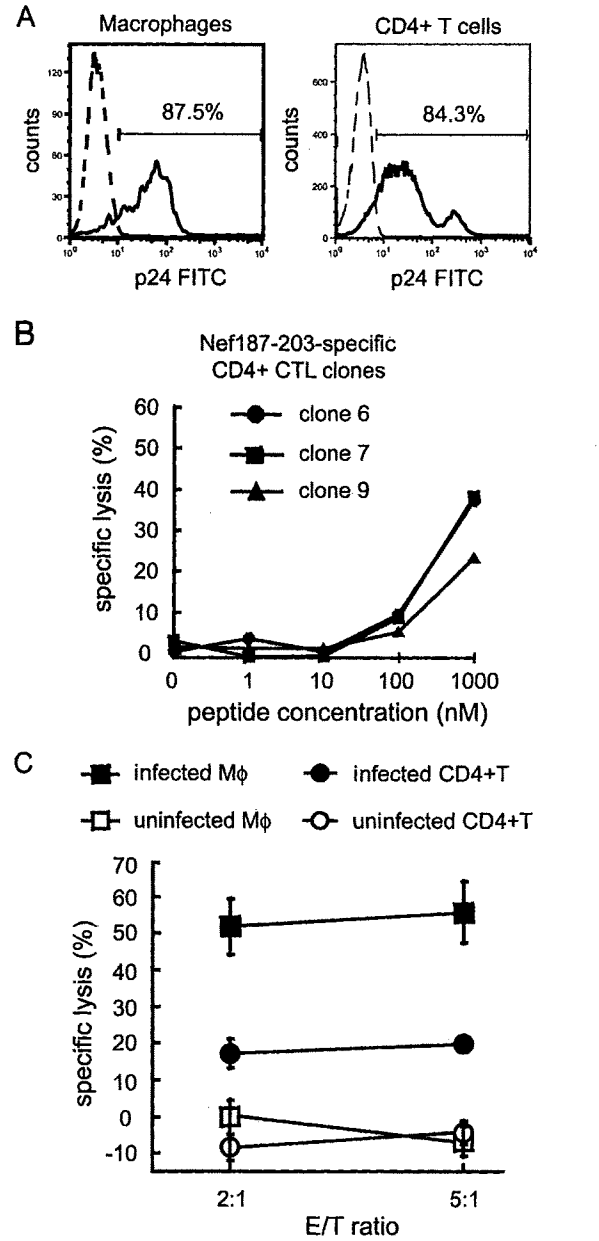

FIG. 4. Lysis of HIV-1-infected macrophages and CD4+ T cells by Nef187-203-specific cytotoxic CD4+ T cells. (A) Intracellular p24 antigen expression of macrophages and CD4+ T cells from an HLA-DRB1*0803-positive donor at day 3 postinfection. The dashed histogram represents uninfected cells, and the solid histogram represents HIV-1-infected cells. The values in each plot show the frequencies of p24 antigen-positive cells. The uninfected and HIV-1-infected macrophages and CD4+ T cells were then labeled with $Na_2{}^{51}CrO_4$ and incubated with Nef187-203-specific clones for CTL assays. (B) Cytotoxic activity of three Nef187-203-specific clones against autologous B-LCLs incubated with the peptide at the indicated concentrations. The cells were tested at an effector-to-target (E/T) ratio of 5:1. (C) Ability of Nef187-203-specific clones to lyse HIV-1-infected or uninfected macrophages (Mϕ) and CD4+ T cells. The cells were tested at the indicated E/T ratios by using the standard ^{51}Cr assay. Values represent averages ± standard deviations (error bars) of results from the assays of the three Nef187-203-specific clones.

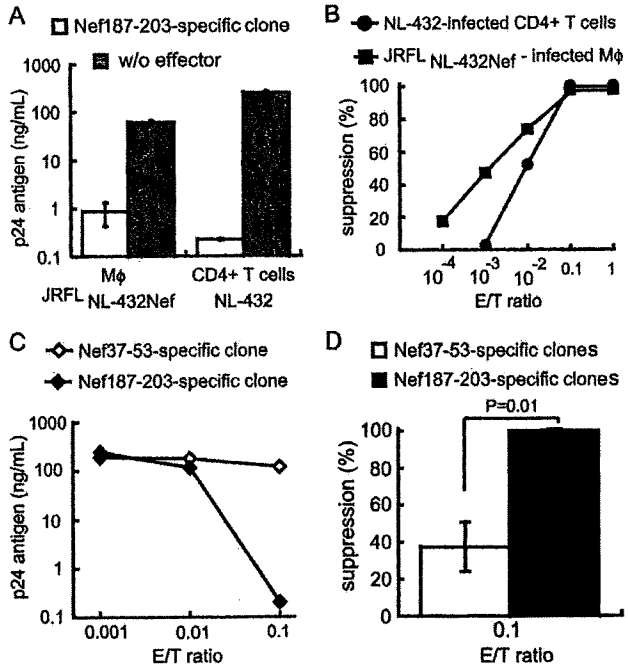

FIG. 5. Ability of HIV-1 cytotoxic CD4$^+$ T cells to suppress HIV-1 replication in vitro. (A) Ability of Nef187-203-specific CD4$^+$ CTL clones to suppress $JRFL_{NL\text{-}432Nef}$ virus and NL-432 virus replication in macrophages and CD4$^+$ T cells, respectively. Macrophages and CD4$^+$ T cells from an HLA-DR-compatible healthy donor were infected with HIV-1 and subsequently cocultured or not with Nef-187-203-specific CD4$^+$ CTL clones at an effector-to-target (E/T) ratio of 0.1:1. The concentration of p24 antigen in the supernatant on day 6 postinfection was measured by using an enzyme immunoassay. Values are presented as the averages ± standard deviations of results from the assays of two Nef187-203-specific clones. (B) The ability of Nef187-203-specific CD4$^+$ CTL clone to suppress HIV-1 infection in target cells was E/T ratio dependent. $JRFL_{NL\text{-}432Nef}$-infected macrophages or NL-432-infected CD4$^+$ T cells were subsequently cocultured with a Nef187-203-specific clone at the indicated E/T ratios. The concentration of p24 antigen in the supernatant on day 6 postinfection was measured as described above. (C) Ability of a Nef37-53-specific CD4$^+$ T-cell clone with no CTL activity to suppress HIV-1 replication in HIV-1-infected CD4$^+$ T cells. CD4$^+$ T cells from two healthy donors expressing the corresponding HLA-DR alleles were infected with HIV-1 and were subsequently cocultured with a Nef37-53-specific or Nef187-203-specific clone at the indicated E/T ratios. The concentration of p24 antigen in the supernatant on day 6 postinfection was measured as described above. (D) The ability of Nef37-53-specific CD4$^+$ T-cell clones to suppress HIV-1 replication in HIV-1 infected CD4$^+$ T cells was less than that of Nef187-203-specific CD4$^+$ CTL clones. Values are presented as averages ± standard deviations (error bars) of results from the assays of three Nef37-53-specific or Nef187-203-specific clones. Statistical differences were determined with Student's t test, and the double-sided P value is shown.

was detected at a low E/T ratio of 0.1:1, indicating that these Nef-specific CD4$^+$ CTLs had a very strong ability to suppress HIV-1 replication. To investigate if this strong suppressor ability could be attributed to the cytolytic activity of CD4$^+$ T cells, we compared the suppressor ability of Nef37-53-specific CD4$^+$ T cells, which did not show significant CTL activity, with that of the Nef187-203-specific CTL clones. A Nef37-53-specific clone with no CTL activity revealed weak suppression activity at an E/T ratio of 0.1:1 against the HIV-1-infected CD4$^+$ T cells from an HLA-compatible healthy donor (Fig. 5C), with this ability being significantly lower than that of the Nef187-203-specific CD4$^+$ CTL clone (Fig. 5D). This result indicates that the Nef-specific cytotoxic CD4$^+$ T cells have strong ability to suppress HIV-1 replication and that noncytotoxic Nef-specific CD4$^+$ T cells may have weak ability to suppress HIV replication via cytokines or by some other mechanism(s).

Detection of Nef187-203-specific CD4$^+$ T cells in chronically HIV-1-infected individuals. To investigate if CD4$^+$ T cells specific for Nef187-203 could be frequently found in HLA-DRB1*0803-positive HIV-1-infected individuals, we expanded our investigation to include nine more chronically HIV-1-infected patients carrying the HLA-DRB1*0803 allele. PBMCs from these patients and KI-197 were stimulated with Nef187-203 peptide and cultured for 2 weeks to expand the population of epitope-specific CD4$^+$ T cells. IFN-γ-producing cells were determined by intracellular staining after restimulation of the bulk cultures with HLA-DRB1*0803-positive B-LCLs prepulsed with the peptide. We observed Nef187-203-specific CD4$^+$ T cells in the bulk cultures from three of these nine donors, i.e., KI-105, KI-121, and KI-154. Taken together, our data indicate that Nef187-203-specific CD4$^+$ T cells were detected among cultured PBMCs from 4 of 10 HLA-DRB1*0803-positive HIV-1-infected individuals (Table 1).

Among the PBMCs from donors KI-154 and KI-197, who showed strong CD4 responses tested by the assay using in vitro-cultured PBMCs, we also detected Nef187-203-specific CD4$^+$ T cells directly ex vivo (Table 1). Furthermore, more than 50% of the Nef187-203-specific CD4$^+$ T cells from both KI-154 and KI-197 mobilized CD107a after stimulation with Nef187-203 peptide (Table 1), demonstrating the existence of cytotoxicity-associated degranulation of Nef187-203-specific CD4$^+$ T cells in these two HIV-1-infected patients.

DISCUSSION

Previous studies showed that Gag and Nef are immunodominant proteins of HIV-1-specific CD4$^+$ T-cell responses in patients at various stages of an HIV-1 infection. Such studies also

TABLE 1. Detection of Nef187-203-specific CD4$^+$ T cells in chronically HIV-1-infected individuals

Subject	HAART	CD4 count (cells/ml)	Viral load (RNA copies/ml)	Frequency (%) of:		
				CD4$^+$ IFN-γ^{+a} cells in:		CD4$^+$ IFN-γ$^+$ CD107a$^+$ cells in ex vivo PBMCs^b
				Cultured PBMCs	Ex vivo PBMCs	
KI-097	+	322	14,000	0	NT^c	NT
KI-105	+	485	<50	2.2	0	0
KI-121	−	265	24,000	18.4	0	0
KI-139	+	505	110,000	0	NT	NT
KI-144	+	496	17,000	0	NT	NT
KI-152	+	303	<50	0	NT	NT
KI-154	+	481	7,700	70.3	0.01	70.0
KI-163	+	419	26,000	0	NT	NT
KI-185	+	331	<50	0	NT	NT
KI-197	+	350	<50	60.7	0.06	55.0

[a] IFN-γ$^+$, IFN-γ-producing.
[b] Frequency among Nef187-203-specific CD4$^+$ IFN-γ$^+$ cells.
[c] NT, not tested.

revealed that only a limited number of peptides may induce CD4 T-cell responses in a genetically diverse population (1, 27). In the present study, we found two Nef $CD4^+$ T-cell epitopes, Nef37-53 and Nef187-203, from two HIV-1-seropositive donors. A previous study showed that a group of subjects with CD4 T-cell responses targeted the peptide Nef187-203; however, the MHC-II restriction of it was not reported (27). Here we characterized both Nef epitopes as HLA-DR restricted in our subjects. Classically, HLA class II-restricted epitopes are processed through the exogenous pathway. However, for $CD4^+$ T-cell recognition of virus-infected cells, the endogenous pathway for HLA class II presentation was also identified in some virus infections (20, 33, 35). In our present study, Nef-specific $CD4^+$ T-cell clones recognized the epitope presented in recombinant vaccinia virus-infected or Nef-GFP fusion mRNA-transfected B-LCLs through the endogenous pathway as well as through the classical exogenous pathway in the antigen protein-pulsed B-LCLs. Furthermore, Nef187-203-specific $CD4^+$ CTLs recognized HIV-1-infected macrophages and $CD4^+$ T cells, suggesting that these HIV-1 host cells could present Nef protein to MHC-II molecules through the endogenous pathway during an HIV-1 infection. Thus, we demonstrated for the first time both endogenous and exogenous presentation of an HIV-1 CD4 epitope by HLA class II molecules.

Since previous studies showed that Gag-specific $CD4^+$ T cells exhibit cytotoxic activity (4, 30, 31), here also we investigated if the same mechanism exists for another immunodominant HIV-1 antigen, Nef. Strong cytotoxic activity was found in the Nef187-203-specific clones in our present study. Compared with the noncytotoxic Nef37-53-specific clone, the cytotoxic Nef187-203-specific clone showed higher perforin and granzyme B expression levels. Although Th clones can acquire cytotoxic behavior during in vitro culture (15), ex vivo studies have directly indicated the persistence of HIV-1-specific cytotoxic $CD4^+$ T cells (31). In addition, a significantly higher perforin expression in a $CD4^+$ subset of PBMCs from HIV-1-infected patients was also observed earlier, suggesting a high prevalence of cytotoxic $CD4^+$ T cells during an HIV-1 infection (4). In our present study, it is unlikely that the observed Nef-specific cytolysis was an artifact of prolonged culture, because ex vivo analysis showed that Nef187-203-specific $CD4^+$ T cells from two donors mobilized CD107a after stimulation with Nef187-203 peptide. Our observations on the cytotoxic effector molecule expression of Nef-specific $CD4^+$ CTL clones suggest that these CTLs kill their target cells by a perforin-dependent pathway, just as in the case of the Gag-specific $CD4^+$ CTLs reported previously (31). The perforin expression in HIV-1-specific $CD4^+$ T cells may be controlled by the CD8 responses during an infection, producing cross-regulation between HIV-1-specific $CD4^+$ and $CD8^+$ T-cell responses (47).

Although Gag-specific $CD4^+$ CTLs were demonstrated to be able to suppress HIV-1 replication in a $CD4^+$ T-cell line, MT-2 (31), the ability of HIV-1-specific $CD4^+$ CTLs to kill infected natural target cells and to suppress HIV-1 replication in these cells has not been explored. $CD4^+$ T cells under normal conditions do not express any HLA class II molecules. Naturally, HIV-1 can replicate only in activated $CD4^+$ T cells, which express MHC-II and are susceptible to $CD4^+$ CTL killing (22). However, the question as to whether the levels of HLA class II expression on HIV-1-infected activated T cells are high enough for efficient recognition by $CD4^+$ CTLs remains unresolved. In addition, previous studies revealed differential susceptibility to $CD8^+$ CTL killing between HIV-1-infected macrophages and $CD4^+$ T cells, showing the complexity of CTL killing of natural target cells during an HIV-1 infection (12, 17, 40). Here we demonstrated higher specific lysis of infected macrophages by Nef-specific $CD4^+$ CTLs than of infected $CD4^+$ T cells by these cells. This result implies that HIV-1-infected macrophages can present virus antigen to HLA class II molecules more effectively than HIV-1-infected $CD4^+$ T cells. On the other hand, naturally higher HLA class II expression on macrophages may also contribute to more-efficient killing of them by $CD4^+$ CTLs. We observed significant HLA class II downregulation on HIV-1-infected $CD4^+$ T cells but not on the infected macrophages (data not shown), in line with a previous report indicating that HIV-1 proteins impair HLA class II expression on infected $CD4^+$ T cells (26). These findings, taken together, may explain why Nef-specific $CD4^+$ CTLs killed HIV-1-infected macrophages more efficiently than HIV-1-infected $CD4^+$ T cells.

Although a difference between cytotoxic activity against HIV-1-infected macrophages and that against $CD4^+$ T cells was observed, Nef-specific $CD4^+$ CTL clones exhibited complete suppression of HIV-1 replication in both kinds of host cells, even at an initial E/T ratio of 0.1 in the assay. The Nef187-203-specific $CD4^+$ CTL clones exhibited a more than 10-fold-stronger ability to suppress HIV-1 replication in macrophages or $CD4^+$ T cells than Nef- or Gag-specific $CD8^+$ CTL clones investigated in our previous studies (17, 18), which employed the same assays, suggesting that Nef187-203-specific $CD4^+$ T cells may be capable of suppressing HIV-1 replication in vivo. In principle, HIV-1-specific T-cell clones can suppress virus replication in two ways: by suppressing cytotoxic activity and cytokine production. A recent study showed that in vitro-cultured noncytotoxic $CD4^+$ T cells produced CCR5 chemokines to suppress HIV-1 replication in those cells themselves (28). In our study, the high level of Mip-1β production by Nef187-203-specific $CD4^+$ CTL clones (data not shown) might also have partly contributed to the suppression of virus replication.

Classically, virus-specific $CD4^+$ T cells play a key role in the maintenance of $CD8^+$ CTL memory (24, 42). In the present study, we sought to demonstrate roles of Nef-specific $CD4^+$ CTLs beyond such helper functions. Notably, we found the suppression of HIV-1 replication in host macrophages and $CD4^+$ T cells by Nef-specific $CD4^+$ CTL clones. Previous investigations showed macrophages to be major reservoirs for HIV-1 in an early infection and in patients with an undetectable viral load on HAART (13). Furthermore, HIV-1-infected macrophages mediate infection of nonlymphoid tissues such as lung or brain (43). Therefore, the strong ability of Nef-specific $CD4^+$ CTLs to suppress HIV-1 replication in macrophages might help to control HIV-1 rebound in structured treatment interruption patients and to relieve the neuropathology associated with AIDS. In addition, Nef-specific $CD4^+$ CTLs may target HIV-infected host cells that resist $CD8^+$ CTL recognition due to an impaired HLA class I antigen-processing pathway. Studies on EBV-specific $CD4^+$ CTLs indicated that they killed EBV-positive Burkitt's lymphoma cells, which are resistant to $CD8^+$ CTL killing, through impaired MHC-I antigen

presentation (2, 37). Thus, particularly in the tissues that can express HLA class II molecules, such as dendritic cells, macrophages, and activated T cells, HIV-1-specific $CD4^+$ CTLs may take the position left vacant due to escape from $CD8^+$ CTL surveillance. However, $CD4^+$ CTLs can also target the antigen-presenting cells and bystander $CD4^+$ T cells, which present epitope peptides through the exogenous pathway. As mentioned by Norris et al. (31), this effect may result in depletion of healthy immune cells during an HIV infection. These results, taken together, indicate that the influence of $CD4^+$ CTLs in vivo on the disease development of AIDS requires more consideration. The frequency of HLA-DR0803-positive patients that responded to the Nef187-203 epitope assessed in our study was 40%, although this value probably was underestimated because previous reports showed that some patients might lose CD4 responses specific for HIV-1 antigens due to vigorous HIV-1 reproduction (7). Exact assessment of the frequency of HIV-1-specific $CD4^+$ T cells would require the use of more-sensitive and cytokine/cytotoxicity response-independent techniques, such as those involving MHC-II tetramers (21).

Overall, our results demonstrated that Nef-specific cytotoxic $CD4^+$ T cells killed HIV-1-infected $CD4^+$ T cells and macrophages in a perforin-mediated manner and that the cytotoxic $CD4^+$ T cells exhibited strong ability to suppress HIV-1 replication in the natural host cells. In addition, our ex vivo analysis revealed that these cytotoxic $CD4^+$ T cells could be detected in 20% of the chronically HIV-infected patients tested. These results, taken together, suggest the importance of Nef-specific $CD4^+$ T cells in the control of HIV-1 infections in vivo.

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Impact of Intrinsic Cooperative Thermodynamics of Peptide-MHC Complexes on Antiviral Activity of HIV-Specific CTL[1]

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The antiviral activity of HIV-specific CTL is not equally potent but rather is dependent on their specificity. But what characteristic of targeted peptides influences CTL antiviral activity remains elusive. We addressed this issue based on HLA-B35-restricted CTLs specific for two overlapping immunodominant Nef epitopes, VY8 (VPLRPMTY) and RY11 (RPQVPLRPMTY). VY8-specific CTLs were more potently cytotoxic toward HIV-infected primary CD4$^+$ cells than RY11-specific CTLs. Reconstruction of their TCR revealed no substantial difference in their functional avidity toward cognate Ags. Instead, the decay analysis of the peptide-MHC complex (pMHC) revealed that the VY8/HLA-B35 complex could maintain its capacity to sensitize T cells much longer than its RY11 counterpart. Corroboratively, the introduction of a mutation in the epitopes that substantially delayed pMHC decay rendered Nef-expressing target cells more susceptible to CTL killing. Moreover, by using differential scanning calorimetry and circular dichroism analyses, we found that the susceptible pMHC ligands for CTL killing showed interdependent and cooperative, rather than separate or sequential, transitions within their heterotrimer components under the thermally induced unfolding process. Collectively, our results highlight the significant effects of intrinsic peptide factors that support cooperative thermodynamics within pMHC on the efficient CTL killing of HIV-infected cells, thus providing us better insight into vaccine design. *The Journal of Immunology,* **2009, 182: 5528–5536.**

Human CD8$^+$ CTLs recognize HIV-infected cells by interaction of their own TCRs with viral peptides bound to HLA class I molecules on the cell surface of the infected cells and eliminate them directly by cytolysis or indirectly through the production of soluble factors such as cytokines and chemokines. Among these activities, the cytotoxic activity of CTLs toward HIV-infected cells is associated with efficient viral containment in vitro and in vivo (1–3). However, significant differences exist not only in the antiviral activity of HIV-specific CTLs among specificities (4–7) but also in CTL specificities between early and chronic phases of an HIV infection (8–10). Changes in CTL specificity could lead to the accumulation of less effective antiviral CTLs in the late chronic phase of an infection (6, 11, 12). There are a number of different possibilities in the literature that potentially explain the heterogeneity in the antiviral activity of CTLs, such as: differences in functional avidity of CTLs toward exogenously pulsed synthetic peptides (7, 13), TCR usage (14, 15), cross-reactive capacity of CTLs toward variant Ags (14, 16), kinetics and amplitude of immunogenic protein expression (9, 17–19), Ag processing and presentation pathways (20, 21), and binding activity of an antigenic peptide to a given HLA class I molecule (22). However, considering that immunodominant peptides are not always those with the highest density presented at the target cell surface (23, 24) and that immunodominant CTLs are not always correlated with effective antiviral CTL responses (25), an interesting question can be raised as to whether, and if so what, inherent characteristics of target epitope peptides support the efficient recognition by CTLs for the killing of virus-infected cells. As mentioned above, however, the antiviral activity of CTLs stems from multifactorial events, reflecting the consequence of various positive and negative factors that govern viral replication, Ag presentation, and T cell activation (26). Broad comparisons between very different virus strains, peptide Ags, and MHCs provide little information beyond highlighting just the differences. Comparisons between more closely related viral Ags and MHCs could be more revealing.

We previously reported that CD8 T cells specific for an Nef epitope (VY8, VPLRPMTY) were consistently elicited very early in vivo, whereas those specific for another Nef epitope (RY11, RPQVPLRPMTY) were mostly observed in the chronic phase of an HIV infection (10). Remarkably, VY8 is entirely contained within RY11; and both are presented by HLA-B35 with comparable binding activity, as assessed by a cellular HLA stabilization assay (10). As initial preliminary experiments showed that VY8-specific CTLs had more potent cytotoxic activity toward HIV-infected primary CD4$^+$ cells than RY11-specific CTLs, in the present study we asked what property of these antigenic peptides is correlated with CTLs having potent antiviral cytotoxic activity. Combining a series of data obtained from T cell lines transduced with the genes for the cognate TCRs, we discovered that the decay of peptide-MHC class I complex (pMHC),[3] rather than the functional avidity of TCR-pMHC interactions, substantially influenced the susceptibility of HIV-infected cells to CTL

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[3] Abbreviations used in this paper: pMHC, peptide-MHC class I complex; DSC, differential scanning calorimetry; CD, circular dichroism; β_2m, β_2-microglobulin.