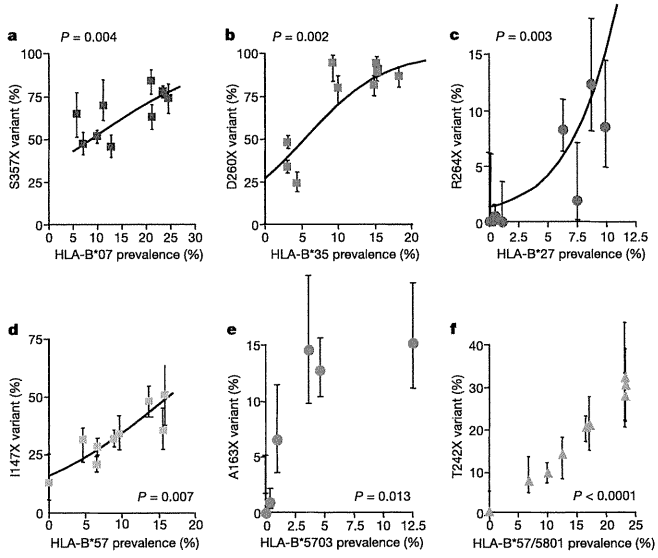

Figure 3 | Correlation between frequency of HIV sequence variant and HLA prevalence for six additional well-characterized epitopes. *P* values calculated after logistic regression analysis as shown (calculations after linear regression analysis are shown in Supplementary Table 1). **a**, Frequency of the S357X mutation within the HLA-B*07-restricted epitope GPSHKARVL (Gag 355–363). **b**, Frequency of the D260X mutation within the HLA-B*35-restricted epitope PPIPVGDIY (Gag 254–262). **c**, Frequency of the R264X mutation within the HLA-B*27-restricted epitope KRWIILGLNK (Gag 263–272). **d**, Frequency of the I147X mutation within the HLA-B*57-restricted epitope ISPRTLNAW (Gag 147–155). **e**, Frequency of the A163X mutation associated with the HLA-B*5703-restricted epitope KAFSPEVIPMF (Gag 162–172). **f**, Frequency of the T242X mutation within the B*57/5801-restricted epitope TSTLQEQIAW (Gag 240–249). Error bars represent 95% confidence limits, obtained using a binomial error distribution.

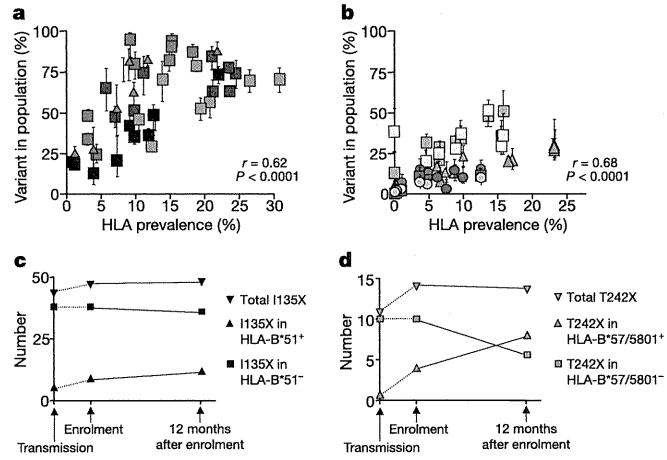

Figure 4 | Correlation between HIV variant frequency and HLA prevalence for all epitopes studied. **a**, Correlation between HLA prevalence and the five stable, non-reverting variants (symbols in Figs 2 and 3, and Supplementary Fig. 3; grey triangles, I31V; green squares, D312X). **b**, Eight variants demonstrated to reduce viral fitness (see text, Fig. 3 and Supplementary Fig. 3; turquoise triangles, L268X; yellow squares. A146X; sky-blue squares, V168I; yellow circles, I247X). **c**, **d**, Data from acute London cohort. **c**, Number of HLA-B*51-positive and HLA-B*51-negative subjects carrying the non-reverting I135X variant. The percentage of I135X in HLA-B*51-negative subjects at enrolment (42%) assumed the percentage of I135X in all subjects at transmission (I135X frequency in HLA-B*51-positive subjects at enrolment was 69%, $P = 0.07$). **d**, The reverting HLA-B*57/5801-restricted T242X mutation. T242X frequency in HLA-B*57/5801-negative subjects at enrolment was 7%, versus 33% in HLA-B*57/5801-positive subjects ($P = 0.01$). Error bars represent 95% confidence limits, obtained using a binomial error distribution.

remained significant even when comparing HLA prevalence with variant frequency in the HLA-mismatched population ($r = 0.40$, $P = 0.0004$). As anticipated, non-reverting variants such as I135X accumulate at the population level, but even rapidly reverting[18,20] mutations such as T242N can accumulate, if the selection rate exceeds the reversion rate (Fig. 4c, d).

Although frequency of the analysed HIV polymorphisms and HLA prevalence were strongly correlated overall, some anomalies were observed. For example, despite a 0% prevalence of HLA-B*57 in Japan[10], 38% of the Japanese cohort had the HLA-B*57-associated A146X variant. One potential explanation might be A146X selection by non-HLA-B*57 Japanese alleles. Analysing Gag sequences from Japanese study subjects, we observed a strong association between A146P and HLA-B*4801 ($P = 0.00035$), and then that A146P is indeed selected in HLA-B*4801-positive subjects (Supplementary Fig. 4a, b). We defined a novel HLA-B*4801-restricted epitope (Gag 138–147), showing also that A146P is an escape mutant (Supplementary Fig. 4c–f). These data illustrate that more than one HLA allele can drive the selection of a particular escape mutant (Supplementary Fig. 5). Also, in populations where HIV-specific $CD8^+$ T-cell responses are incompletely characterized, the influences of locally prevalent HLA alleles on HIV sequence variation are unknown.

These data show a strong correlation between HLA-associated HIV sequence variation and HLA prevalence in the population ($r = 0.69$, $P < 0.0001$, Supplementary Fig. 6), suggesting that the frequency of the studied variants is substantially driven by the HLA-restricted $CD8^+$ T-cell responses. Non-reverting variants[5,7], as well as those previously shown to arise at a fitness cost[7,14,16–21], were studied. The latter constitute approximately 55–65% of HLA-associated polymorphisms[7,20]. This current analysis included epitopes whose role in HIV immune control is unknown, as well as those believed to contribute significantly to containment of HIV[4,7,13–19]. Analysis of well-characterized epitopes only also served to limit potential confounding influences of epitope clustering (selection of the same variant by different HLA alleles) and of founder effect. Either would be capable of obscuring a true HLA effect on population variant frequency.

The HLA-B*57-associated A146X mutation illustrates the complexity that may result from epitope clustering. A146X is selected by at least six distinct HLA alleles (Supplementary Fig. 5). A true correlation existing between mutation frequency and individual HLA allele prevalence might thus be obscured by selection of the same mutation by other alleles.

Founder effect also has an undoubted influence on population frequencies of particular polymorphisms[6]. Phylogenetic correction of sequence data excludes founder effect as a confounder[6,7,9], and the highly significant associations between the presence of particular HLA alleles and all 14 HIV polymorphisms studied, persisting after phylogenetic correction (Supplementary Table 3), provide compelling evidence that the effects observed here are substantially HLA-driven. The large numbers of study subjects in these current studies reduce the likelihood of genuine HLA associations with HIV amino acid polymorphisms being obscured by founder effects. The relative impact of HLA and founder effect on variant frequency is harder to quantify, and is likely to differ substantially between particular populations.

The consequence of HIV adapting to certain $CD8^+$ T-cell responses is unknown. For non-reverting polymorphisms such as HLA-B*35-associated D260E, the variant approaches fixation, because even at population frequencies of 90%, D260E is still significantly selected in HLA-B*35-positive subjects (Supplementary Fig. 7b). Important questions relevant to vaccine design include the extent and rate of sequence change in populations. Relevant factors include the selection rate in subjects expressing the HLA allele, the reversion rate in HLA-mismatched subjects, the population HIV

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).

METHODS

Study subjects. The study cohorts have been described more fully elsewhere[3,7,9,13,14,18,20,21,27]. All comprise chronically infected and highly active antiretroviral therapy (HAART)-naive study subjects, with the exception of the London acute cohort ($n = 142$), who were enrolled immediately after seroconversion between 1999 and 2004, and 54 subjects enrolled during acute infection in Japan between 1997 and 2008. Viral sequences in all 2,679 chronically infected study subjects (all of whom were HAART-naive) were determined from time points after 2000, with the exception of 9 study subjects in the Japanese chronic cohort (1998–99) and all of the British Columbia cohort (1996–99). Sequencing data were obtained from 566 study subjects in the British Columbia cohort, 53 study subjects in the Barbados cohort, 106 in the Oxford cohort, 673 in the Durban cohort, 226 in the Lusaka cohort (chronically infected subjects enrolled between 2005–08), 481 study subjects in the Perth cohort, 277 chronically infected subjects in the Kumamoto cohort, 297 in the Gaborone cohort, and 142 subjects in the acute London cohort. An additional cohort in Japan comprised 117 haemophiliacs who were infected before 1985, the majority of which were believed to have been infected in 1983, and who were enrolled and followed up in out-patient clinics since 1997. These haemophiliacs are all now on HAART except for 4 HAART-naive subjects.

HLA-associated HIV amino acid polymorphisms studied. Variants studied that were shown to reduce viral fitness comprised polymorphisms within the HLA-B*27-restricted Gag epitope KRWIILGLNK (Gag 263–272; R264X and L268X) and mutations in three HLA-B*57-restricted Gag epitopes: ISPRTLNAW (ISW9, Gag 147–155), KAFSPEVIPMF (KF11, Gag 162–172) and TSTLQEQIAW (TW10, Gag 240–249). T242X is strongly selected by HLA-B*5801 in addition to HLA-B*57 subtypes[7,14,17]. The HLA-B*57-associated polymorphisms at residues Gag 146, 147 and 248 are selected by all HLA-B*57 subtypes, whereas Gag 163, 165, 166 and 247 are only selected by the HLA-B*5703 subtype (refs 7, 18 and H.C., unpublished data).

Statistics. Polymorphism frequency in the study cohorts was compared with prevalence of the relevant HLA molecule in the study cohort using a logistic regression model. To take account of the different numbers of study subjects in each cohort, appropriate confidence limits for the mutation frequencies were calculated, using the Adjusted–Wald method for binomial variables[28]. Logistic regression was calculated by GLMStat (http://www.glmstat.com) using a binomial error distribution and a logit link function. In addition, the Spearman's rank correlation coefficient was calculated in the context of a linear regression model (data shown in Supplementary Tables 1 and 2).

HLA class I typing. Because HLA typing was not undertaken consistently to four-digit resolution in all cohorts, two-digit HLA types only were used for these analyses, with the exception of the HLA-B*5703-associated polymorphisms (the Barbados and Oxford cohorts being excluded from these latter analyses as HLA-B*57 subtyping data were not available). Genomic DNA samples were initially typed to an oligo-allelic (two-digit) level using Dynal RELITM reverse SSO kits for the HLA-A, HLA-B and HLA-C loci (Dynal Biotech). Refining the genotype to the allele level was performed using Dynal Biotech sequence-specific priming (SSP) kits in conjunction with the previous SSO type. HLA phenotypic frequencies were determined from the HIV-infected study cohorts themselves.

Sequencing of viral RNA and proviral DNA. Viral sequencing of *gag* and *pol* from plasma RNA and proviral DNA was undertaken, using primers as previously described[7,9]. PCR products were sequenced directly or they were cloned by using a TOPO TA cloning kit (Invitrogen) and then sequenced. Sequencing was done with a Big Dye terminator v1.1. cycle sequencing kit (Applied Biosystems) and analysed by an ABI PRISM 310 genetic Analyser.

Competitive HIV-1 replication assay. Freshly prepared H9 cells (3×10^5) were exposed to the mixtures of paired virus preparations (300 blue cell-forming units) each of NL-432 versus mutant virus (I135T, I135V, I135R and I135L)), to be examined for their replication ability for 2 h, washed twice with PBS, and cultured as described previously[29]. On day 1, one-third of infected H9 cells were harvested and washed twice with PBS, and the proviral HIV-1 reverse transcriptase gene was sequenced (0 week). Every 7 days, the supernatant of the virus culture was transmitted to new uninfected H9 cells. The cells harvested at the end of every other passage (that is, at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 weeks) were subjected to direct DNA sequencing of the HIV-1 reverse transcriptase gene, and the viral population change was determined by the relative peak height on the sequencing electrogram. The persistence of the original amino acid substitution was confirmed for all infectious clones used in this assay.

HLA-B*5101 stabilization assay. Binding of HIV-1-derived peptides to HLA-B*5101 was measured as previously described by using RMA-S-B*5101 cells[8].

Assays to determine recognition of peptide-pulsed or virus-infected targets. C1R and .221 cells expressing HLA-B*5101 or HLA-B*4801 were generated as previously described[30]. All cells were maintained in RPMI 1640 medium supplemented with 10% FCS and 0.15 mg ml^{-1} hygromycin B. Cytotoxicity of CD8$^+$ T cells for C1R-B*5101 cells pre-pulsed with peptide measured by the standard ^{51}Cr release assay was as previously described[8]. .221-B*4801 and .221 cells infected with NL4-3 or NL4-3 A146P mutant virus were used as target cells for intracellular cytokine staining assay.

Generation of the NL4-3 A146P mutant virus. The p82-2 plasmid containing the A146P mutation[4] was digested with BssHII and ApaI. The BssHII–ApaI 1.3-kb fragment was purified and then ligated into the same site of BssHII–ApaI-digested pNL-432 plasmid. To obtain pNL-432 including the A146P mutant (pNL-432 A146P), 293T cells were transfected with pNL-432 A146P using Lipofectamine 2000 (Invitrogen). Supernatants from transfected 293T cell cultures were stored at −80 °C.

Generation of CD8$^+$ T-cell clones and peptide-specific CD8$^+$ T-cell lines. Cytotoxic T lymphocyte (CTL) clones were generated from HIV-1-specific bulk-cultured T cells by limiting dilution as previously described[8]. Peptide-specific CD8$^+$ T-cell lines were generated by stimulating peripheral blood mononuclear cells (PBMCs) from the HLA-B*4801-positive HIV-1-seropositive individual KI-092 with the NI11 (NLQGQMVHQAI) peptide and then culturing them for 2 weeks[8]. Cytotoxicity of CD8$^+$ T cells for target cells pre-pulsed with peptide measured by the standard ^{51}Cr release assay was as previously described[8].

Suppression assay of HIV-1 replication by HIV-1-specific CTLs. The ability of HIV-1-specific CTLs to suppress HIV-1 replication was examined as previously described[30].

Intracellular cytokine staining assays. PBMCs from HIV-1-infected individuals were stimulated with the desired peptide (1 μM) and cultured for 12–14 days. These cultured PBMCs were assessed for IFN-γ-producing activity as previously described[30].

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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

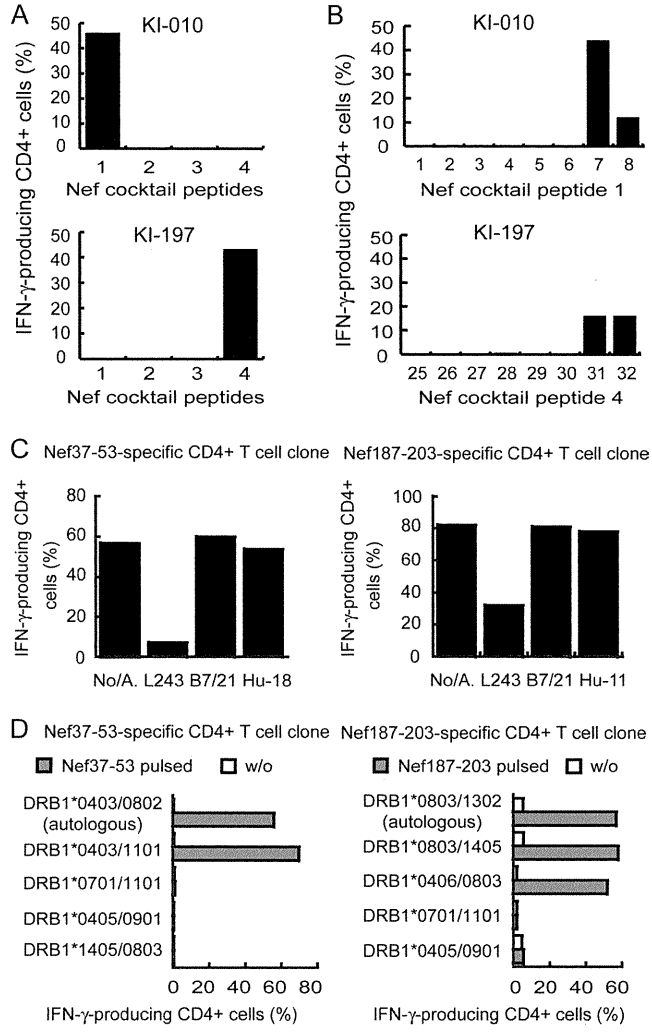

FIG. 1. Identification and characterization of two HIV-1 Nef-specific $CD4^+$ T-cell epitopes. (A) Induction of Nef-specific $CD4^+$ T cells from PBMCs of HIV-1-infected individuals. PBMCs from two HIV-1-seropositive donors (KI-010 and KI-197) were stimulated with cocktails comprising eight 17-mer overlapping 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.

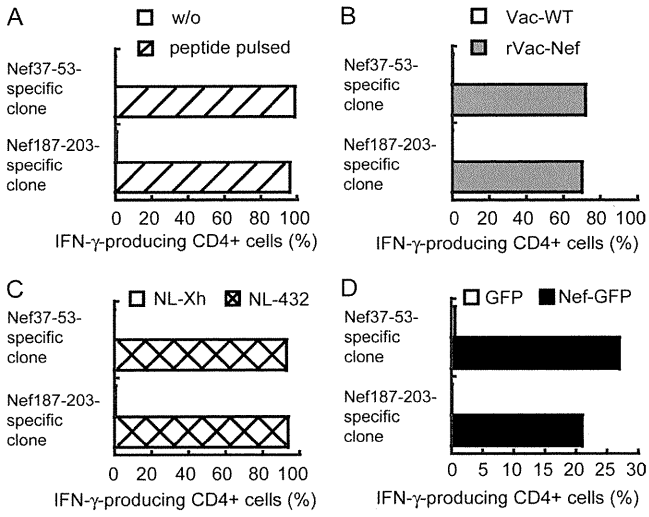

FIG. 2. Naturally occurring presentation of $CD4^+$ T-cell epitopes. Nef37-53-specific and Nef187-203-specific $CD4^+$ T-cell clones were stimulated with peptide-pulsed, recombinant vaccinia virus-infected, HIV-1 particle protein-pulsed, or Nef-GFP fusion mRNA-transfected autologous B-LCLs. The percentage 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

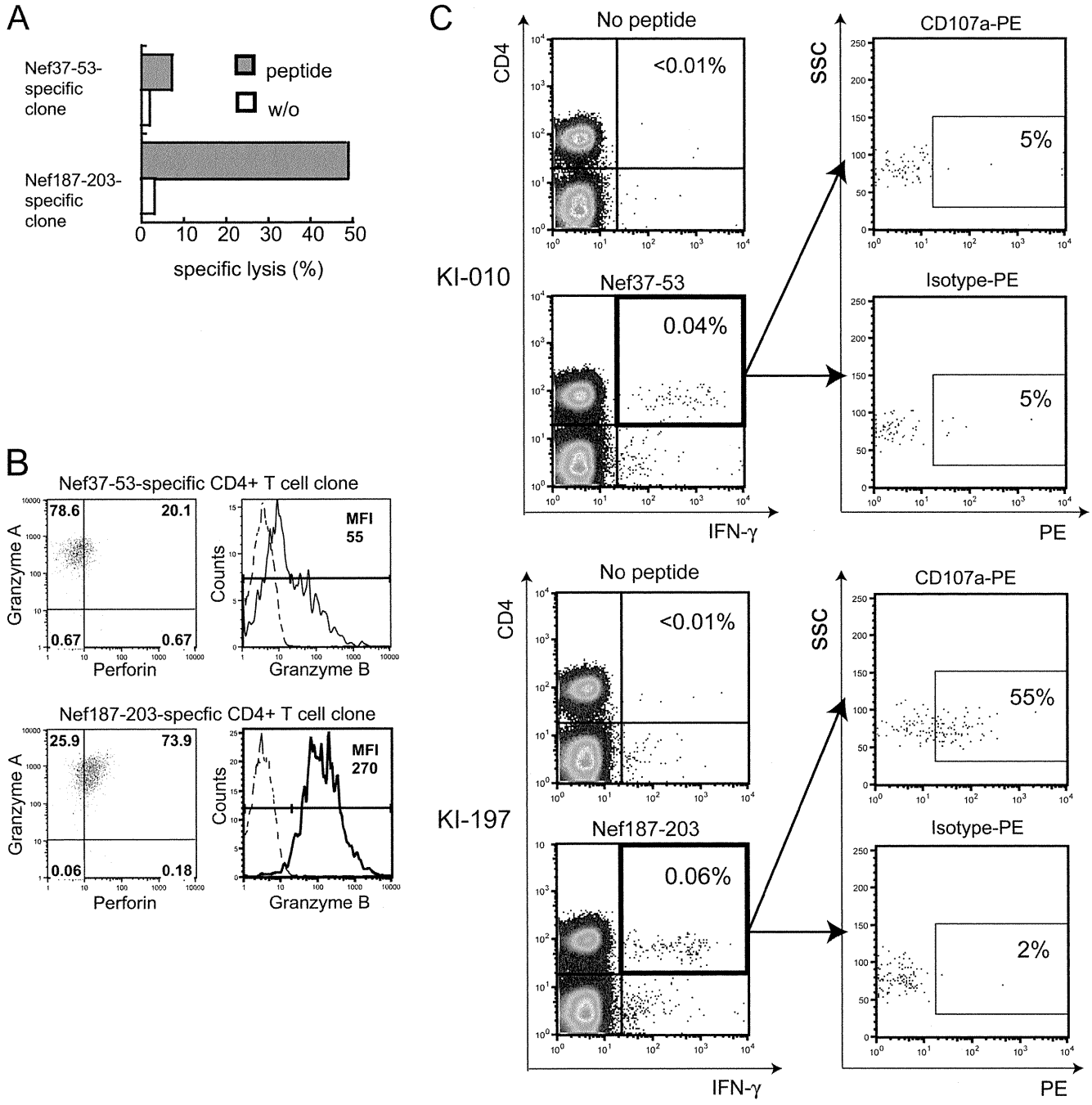

FIG. 3. Cytotoxic activity and cytotoxic effector molecule expression of HIV-1 Nef-specific $CD4^+$ T cells. (A) Cytotoxic activities of a KI-010-derived Nef37-53-specific $CD4^+$ T-cell clone and a KI-197-derived Nef187-203-specific $CD4^+$ T-cell clone against autologous B-LCLs incubated with the epitope 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

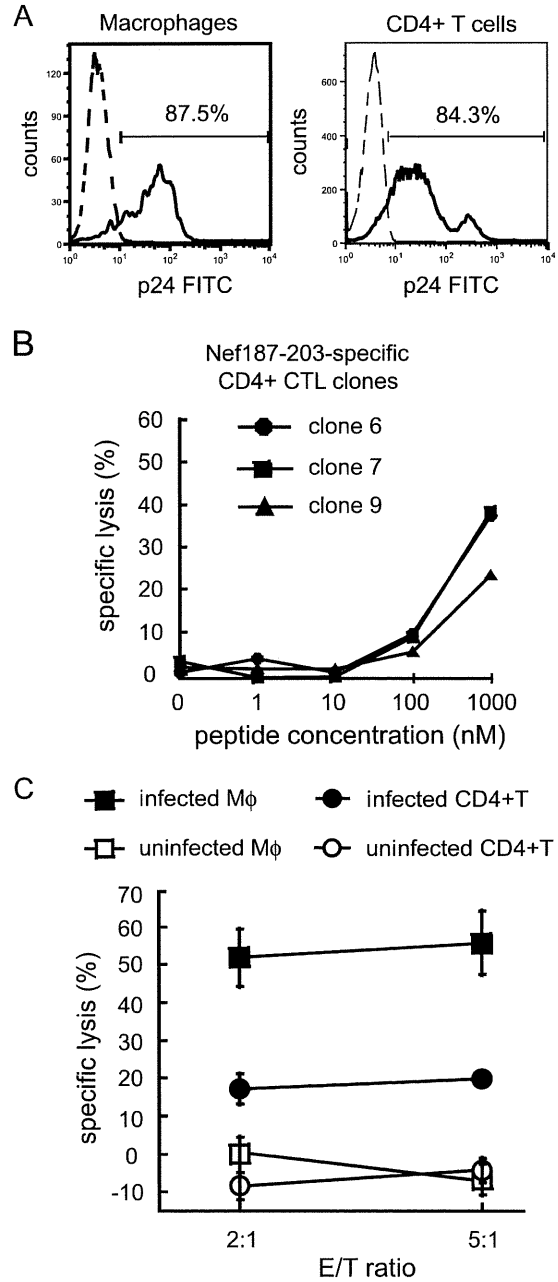

FIG. 4. Lysis of HIV-1-infected macrophages and CD4+ T cells by Nef187-203-specific cytotoxic CD4+ T cells. (A) Intracellular p24 antigen expression of macrophages and CD4+ T cells from an HLA-DRB1*0803-positive donor at day 3 postinfection. The dashed histogram represents uninfected cells, and the solid 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.

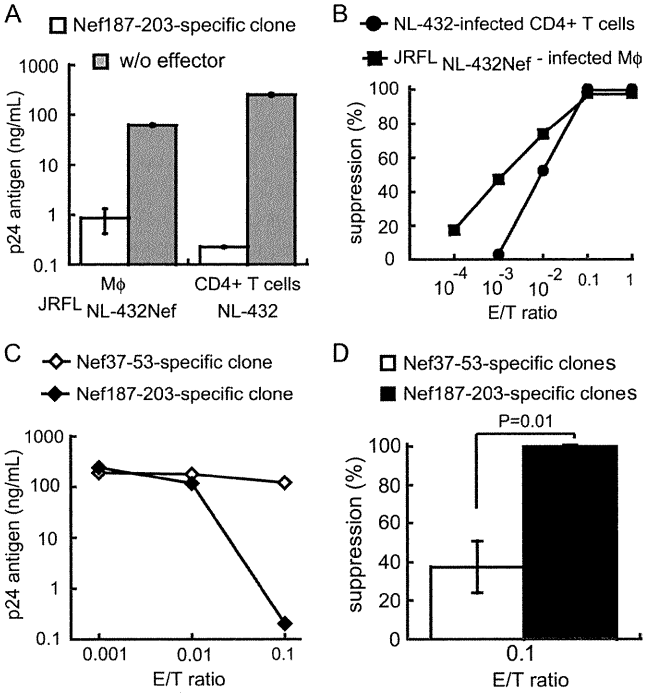

FIG. 5. Ability of HIV-1 cytotoxic CD4$^+$ T cells to suppress HIV-1 replication in vitro. (A) Ability of Nef187-203-specific CD4$^+$ CTL clones to suppress $JRFL_{NL\text{-}432Nef}$ virus and NL-432 virus replication in macrophages and CD4$^+$ T cells, respectively. Macrophages and CD4$^+$ T cells from 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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Different *In Vivo* Effects of HIV-1 Immunodominant Epitope-Specific Cytotoxic T Lymphocytes on Selection of Escape Mutant Viruses▽‡

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HIV-1 escape mutants are well known to be selected by immune pressure via HIV-1-specific cytotoxic T lymphocytes (CTLs) and neutralizing antibodies. The ability of the CTLs to suppress HIV-1 replication is assumed to be associated with the selection of escape mutants from the CTLs. Therefore, we first investigated the correlation between the ability of HLA-A*1101-restricted CTLs recognizing immunodominant epitopes *in vitro* and the selection of escape mutants. The result showed that there was no correlation between the ability of these CTLs to suppress HIV-1 replication *in vitro* and the appearance of escape mutants. The CTLs that had a strong ability to suppress HIV-1 replication *in vitro* but failed to select escape mutants expressed a higher level of PD-1 *in vivo*, whereas those that had a strong ability to suppress HIV-1 replication *in vitro* and selected escape mutants expressed a low level of PD-1. *Ex vivo* analysis of these CTLs revealed that the latter CTLs had a significantly stronger ability to recognize the epitope than the former ones. These results suggest that escape mutations are selected by HIV-1-specific CTLs that have a stronger ability to recognize HIV-1 *in vivo* but not *in vitro*.

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 (5, 28, 33). On the other hand, HIV-1 can escape from the host immune system by various mechanisms. These may include the appearance of HIV-1 carrying escape mutations in its immunodominant CTL epitopes as well as Nef-mediated downregulation of HLA class I molecules. There is a growing body of evidence for the former mechanism, i.e., that CTLs targeting immunodominant HIV-1 epitopes select escape mutants in chronically HIV-1-infected individuals (18, 20, 36), whereas the latter mechanism was proved by demonstrating that HIV-1-specific CTLs fail to kill Nef-positive-HIV-1-infected $CD4^+$ T cells but effectively kill Nef-defective-HIV-1-infected ones or that they suppress the replication of Nef-defective HIV-1 much more than that of Nef-positive HIV-1 (12, 13, 42, 45).

It is speculated that HIV-1 immunodominant epitope-specific CTLs have the ability to suppress HIV-1 replication and effectively select escape mutants. However, the correlation between this ability of the CTLs and the appearance of escape mutants is still unclear, because it is not easy to evaluate the ability of HIV-1-specific CTLs to exert a strong immune pressure *in vivo*. To examine this ability, most previous studies measured the number of HIV-1-specific CTLs or $CD8^+$ T cells and the CTL activity against target cells prepulsed with the epitope peptide or those infected with HIV-1 recombinant vaccinia virus (6, 7, 23, 46). However, the results obtained from such experiments do not reflect the ability of the CTLs to exert immune pressure *in vivo*. We and other groups previously utilized an assay to directly evaluate the ability of the CTLs to suppress HIV-1 replication *in vitro* (1, 17, 18, 42, 43). This assay may be better for evaluation of immune pressure by HIV-1-specific CTLs than other assays, because the ability of the CTLs to suppress HIV-1 replication is directly measured in cultures of HIV-1-infected $CD4^+$ T cells incubated with HIV-1-specific CTL clones. But it still remains unknown whether this assay reflects immune pressure *in vivo*.

In the present study, we investigated whether HIV-1-specific CTLs having a strong ability to suppress HIV-1 replication could positively select escape mutants. Since HLA-A*1101 is known to be an HLA allele relatively associated with a slow progression to AIDS (32), it is speculated that some HLA-A*1101-restricted CTLs would have a strong ability to suppress HIV-1 replication *in vitro*. Therefore, we first focused on 4 well-known HLA-A*1101-restricted CTL epitopes in the present study. We investigated the frequency of CTLs specific for these epitopes in chronically HIV-1-infected individuals, the ability of these CTLs to suppress HIV-1 replication *in vitro*, and whether the escape mutants were selected by the CTLs. Furthermore, we analyzed the expression of Programmed Death-1 (PD-1) on these CTLs *ex vivo* and antigen recognition of them.

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MATERIALS AND METHODS

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.

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 primers (Invitrogen). The Nef region and the Gag region were 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 by using a BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems) and an ABI Prism 310 genetic analyzer. The regions of Gag349, Nef73, and Nef84 epitopes were sequenced directly in 124, 121, and 122 individuals, respectively, while those of Nef73 and Nef84 epitopes were sequenced for cloned samples from 10 and 11 individuals, respectively.

Cells. C1R cells expressing HLA-A*1101 (C1R-A*1101) and transporter associated with antigen processing (TAP)-defective RMA-S cells expressing HLA-A*1101 (RMA-S-A*1101) were previously generated and were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 0.15 mg/ml hygromycin B.

Generation of CTL clones. Peptide-specific CTL clones were generated from an established peptide-specific bulk CTL culture by seeding 0.8 cell/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 individual, and 1×10^5 irradiated C1R-A*1101 cells prepulsed with a 1 μM concentration of the corresponding peptide, Gag349 [ACQGVG GPGHK], Nef73 [QVPLRPMTYK], or Nef84 [AVDLSHFLK]). 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.

HIV-1 clones. Infectious proviral clones of HIV-1, pNL-432, and its Nef mutant, pNL-M20A (containing a substitution of Ala for Met at residue 20 of Nef), reported previously, were used (2). For pNL-432-Nef84-2L9R, the mutation was introduced by site-directed mutagenesis (Invitrogen).

CTL assay for target cells pulsed with HIV-1 peptide. Cytotoxicity activity was measured by the standard ^{51}Cr release assay, as previously described (34). Target cells (2×10^5) were incubated for 60 min with 100 μCi $Na_2{}^{51}CrO_4$ in saline and then washed three 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.

Intracellular cytokine (ICC) production assay. PBMCs from HLA-A*1101-positive HIV-1-infected patients were stimulated with a given peptide (1 μM) in culture medium (RPMI 1640 medium supplemented with 10% FCS and 200 U/ml recombinant human interleukin-2). After 14 days in culture, the cells were assessed for gamma interferon (IFN-γ) production activity by using a FACSCalibur instrument. Briefly, bulk cultures were stimulated by C1R-A*1101 cells pulsed with or without the corresponding 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 7-amino-actinomycin D (7-AAD) at room temperature for 10 min. After 2 washes with RPMI 1640 medium supplemented with 10% FCS, cells were 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 permeablization buffer, the cells were analyzed by using the FACSCalibur instrument. Nonspecific binding of anti-IFN-γ MAb and nonspecific production of IFN-γ were excluded by subtracting the data of the negative control, which was the same sample stimulated with C1R-A*1101 cells without the specific peptide and stained with the same MAbs.

For *ex vivo* analysis, PBMCs from HLA-A*1101-positive HIV-1-infected patients were stimulated with the corresponding peptide (1 μM), and IFN-γ production was measured 6 h later, as described above.

HLA class I stabilization assay. The binding of peptides to HLA-A*1101 molecules was tested as previously described (11). RMA-S-A*1101 cells transfected with HLA-A*1101 and human β_2-microglobulin were used. These cells express a very low level of HLA class I molecules on their cell surface when they are cultured at 37°C, whereas empty HLA class I molecules are stably expressed if they are cultured at 26°C. The stabilization of HLA class I molecules is dependent on peptide binding affinity (22, 30, 40). Briefly, RMA-S-A*1101 cells were cultured at 26°C for 14 to 18 h. The cells were incubated at 26°C for 1 h with Nef84 (AVDLSHFLK), Nef84-2L (ALDLSHFLK), or Nef84-2L9R (ALDLSHFLR) peptide at various concentrations and then at 37°C for 3 h. After 2 washes with phosphate-buffered saline (PBS) supplemented with 20% FCS (PBS-20% FCS), they were subsequently incubated for 30 min on ice with an appropriate dilution of MAb TP25.99 (41). After 2 washes with PBS-20% FCS, the cells were incubated for 30 min on ice with an appropriate dilution of FITC-conjugated sheep IgG with anti-mouse Ig specificity (Silenus Laboratories, Hawthorn, Australia). Finally, they were washed three times with PBS-20% FCS, after which the fluorescence intensity was measured by using a flow cytometer (Becton Dickinson, Mountain View, CA).

Surface expression of HLA class I molecules on HIV-1-infected cells. To assess HLA class I expression on HIV-1-infected $CD4^+$ T cells, we stained the cells with anti-HLA-A11 MAb followed by PE-labeled anti-mouse Ig (Pharmingen International, San Diego) and thereafter fixed and permeabilized them for intracellular HIV-1 p24 staining with FITC-labeled anti-p24 MAb KC-57. The expression of HLA class I molecules on HIV-1infected $CD4^+$ T cells was examined by using the FACSCalibur instrument with Cell Quest software (Becton Dickinson, San Jose, CA).

Suppression of HIV-1 replication by HIV-1-specific CTL clones. The ability of HIV-1-specific CTL clones to suppress HIV-1 replication was examined as previously described (42). $CD4^+$ T cells purified by means of anti-human CD4 MAb-coated magnetic beads (MACS beads; Miltenyi Biotec) from PBMCs of an HIV-1-seronegative individual with HLA-A*1101 were cultured and infected with HIV-1 clones. Cultured $CD4^+$ T cells were incubated with an HIV-1 clone for 4 h at 37°C with intermittent agitation and then washed three times with RPMI 1640 medium supplemented with 10% FCS. HIV-1-infected $CD4^+$ T cells were cocultured with an HIV-1-specific CTL clone in culture medium. From day 2 to day 7 postinfection, 10 μl of culture supernatant was collected, and the concentration of p24 antigen (Ag) in the supernatant was measured by conducting an enzyme immunoassay (HIV-1 p24 Ag enzyme-linked immunosorbent assay [ELISA] kit; ZeptoMetrix). Percent suppression was calculated as follows: (concentration of p24 Ag in the supernatant of HIV-1-infected $CD4^+$ T cells cultured with HIV-1-specific CTLs/concentration of p24 Ag in the supernatant of HIV-1-infected $CD4^+$ T cells cultured without the CTLs) × 100.

HLA-peptide tetrameric complexes. The tetrameric complexes of HLA-A*1101, HLA-A*2402, and HLA-A*2601 were synthesized as previously described (3). 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.

Analysis of PD-1 or CD27 CD28 CD45RA expression on HIV-1-specific $CD8^+$ T cells. For the analysis of PD-1 expression, cryopreserved PBMCs of HIV-positive individuals were first stained with Pacific Blue-conjugated CD8 MAb (BD Bioscience) and FITC-conjugated CD3 MAb (Dako Corporation, Glostrup, Denmark) at 4°C for 30 min followed by PE-conjugated PD-1 MAb (BD Bioscience) at the room temperature for 30 min. After 2 washes with RPMI 1640 medium supplemented with 10% FCS, the cells were stained with allophycocyanin (APC)-conjugated tetramer at 37°C for 30 min. After 2 additional washes, the cells were stained with 7-AAD (BD Bioscience) at room temperature for 10 min and analyzed by using flow cytometry (FACS Canto II; BD Bioscience). For the phenotypic analysis of HIV-1-specific $CD8^+$ T cells, the PBMCs were first stained with PE-Cy7-conjugated anti-CD3 (BioLegend), Pacific Blue-conjugated CD8 (BD Bioscience), FITC-conjugated anti-CD27 (BD Bioscience), PE-conjugated anti-CD28 (BioLegend), and phycoerythrin-Texas red (ECD)-conjugated anti-CD45RA (Beckman Coulter) MAbs at 4°C for 30 min. After 2 washes with RPMI 1640 medium supplemented with 10% FCS, the cells were stained with APC-conjugated tetramer at 37°C for 30 min. After 2 additional washes, the cells were stained with 7-AAD at room temperature for 10 min and analyzed by using flow cytometry.

Enzyme-linked immunospot (ELISPOT) assay. Cryopreserved PBMCs of 2 HLA-A*1101$^+$ HIV-1-infected individuals (KI-015 and KI-036) were plated out in 96-well polyvinylidene plates (Millipore, Bedford, MA) which had been pre-

coated with 0.5 μg/ml anti-IFN-γ MAb 1-DIK (Matbech, Stockholm, Sweden). The appropriate amount of Nef73 or Nef84 peptides was added in a volume of 50 μl, and then PBMCs were added at 1×10^5 cells/well in a volume of 100 μl. The plate was incubated for 40 h at 37°C in 5% CO_2 and was washed with PBS before the addition of biotinylated anti-IFN-γ MAb (Mabtech) at 0.5 μg/ml. After it was incubated at room temperataure for 100 min and then washed with PBS, streptavidin-conjugated alkaline phospatase (Mabtech) was added following a 40-min incubation at room temperature. Individual cytokine-producing cells were detected as dark spots after a 20-min reaction with 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium by using an alkaline phosphatase-conjugated substrate (Bio-Rad, Richmond, CA). The spot number was counted by using an Eliphoto counter (Minerva Teck, Tokyo, Japan). The number of spots for each peptide-specific T cell response was caluculated by subtracting the negative-control spots.

RESULTS

Immunodominancy of 4 HLA-A*1101-restricted HIV-1 epitopes. We first focused on HIV-1 CTL epitopes presented by only a given HLA allele that influences the control of HIV-1, because the effect of each epitope presented by the same HLA class I allele on the ability of specific CTLs to suppress HIV-1 replication and to select escape mutants can be compared. HLA-A*1101 is an HLA allele relatively associated with a slow progression to AIDS (32), implying that some epitope-specific CTLs may have the ability to suppress HIV-1 replication. We selected 4 out of many known HLA-A*1101-restricted HIV-1 epitopes (Gag349, ACQGVGGPGHK; Pol675, QIIEQLIKK; Nef73, QVPLRPMTYK; and Nef84, AVDLSHFLK; or Nef84-2L, ALDLSHFLK [both sequences are frequently found in clade B]), because CTLs specific for these epitopes were previously shown to be frequently detected in chronically HIV-1-infected individuals (10, 14, 19). We re-evaluated whether $CD8^+$ T cells specific for these HIV epitopes could be frequently detected in chronically HIV-1-infected Japanese individuals carrying HLA-A*1101. PBMC from these individuals and HIV-1-seronegative HLA-$A*1101^+$ individuals were stimulated with these epitope peptides and cultured for 2 weeks. The percentage of specific $CD8^+$ T cells in these cultures was determined by performing an intracellular cytokine (ICC) production assay using these epitope peptides (Fig. 1A). Pol675-specific $CD8^+$ T cells were detected in only 1 of the 8 individuals, whereas Gag349-specific, Nef73-specific, and Nef84- or Nef84-2L-specific ones were detected in 12 of 16 individuals, 13 of 16 individuals, and 11 of 16 individuals, respectively (Fig. 1B). These results indicate that Gag349, Nef73, and Nef84 (or Nef84-2L) are recognized as immunodominant epitopes in HIV-1-infected Japanese individuals carrying HLA-A*1101. We therefore focused on these 3 epitopes for further studies.

Ability of 3 HLA-A*1101-restricted HIV-1-specific CTLs to suppress HIV-1 replication *in vitro*. To investigate the ability of these T cells to suppress HIV-1 replication, we next established 5 Gag349-specific, 7 Nef73-specific, and 3 Nef84-specific CTL clones from PBMC of chronically HIV-1-infected individuals carrying HLA-A*1101. These CTL clones exhibited a strong cytolytic activity against C1R-A*1101 cells prepulsed with the corresponding epitope peptide (Fig. 2A) and against those infected with recombinant vaccinia virus expressing the HIV-1 SF2 Nef or Gag protein (data not shown). We investigated the ability of these CTL clones to suppress HIV-1 replication in primary $CD4^+$ T cells infected with the NL-432 clone or its Nef mutant NL-M20A, which has the ability to downregulate the cell surface expression of CD4 but not that of HLA-class I A and B molecules, in HIV-1-infected cells (2). Indeed, NL-432-infected $CD4^+$ T cells exhibited the downregulation of HLA-A*1101, whereas NL-M20A-infected ones did not (Fig. 2B). Both Nef73-specific and Nef84-specific CTL clones completely suppressed the replication of both NL-432 and NL-M20A at effector/target cell (E:T) ratios of 1:1 and 0.1:1 (Fig. 2C). A Gag349-specific CTL clone partially suppressed NL-432 replication and completely suppressed that of NL-M20A at an E:T ratio of 1:1 but failed to suppress the replication of either clone at an E:T ratio of 0.1:1 (Fig. 2C). Analysis using 6 Gag349-specific, 7 Nef73-specific, and 3 Nef84-specific CTL clones confirmed that the ability of the Nef73-specific and Nef84-specific CTL clones to suppress HIV-1 replication was much stronger that of the Gag349-specific ones (Fig. 2D). It also revealed that Nef-mediated HLA-class I downregulation did not affect the recognition of HIV-1-infected $CD4^+$ T cells by Nef73-specific and Nef84-specific clones. These results together indicate that Nef73-specific and Nef84-specific CTLs have a strong ability to suppress HIV-1 replication *in vitro*.

***Ex vivo* analysis of Nef73-specific and Nef84-specific CTLs in chronically HIV-1-infected individuals.** Nef73-specific and Nef84-specific CTLs could be induced from the memory T-cell pool by *in vitro* stimulation with the specific peptides in more than 50% of chronically HIV-1 infected individuals carrying HLA-A*1101 (Fig. 1). To clarify whether these specific T cells would be elicited *in vivo*, we analyzed PBMCs from chronically HIV-1-infected individuals carrying HLA-A*1101 by using the specific tetramers. Nef73-specific $CD8^+$ T cells were detected for 16 of 20 chronically HIV-1-infected HLA-$A*1101^+$ individuals, and Nef84-specific $CD8^+$ T cells were detected for 13 of 17 (Fig. 3). These results together with those shown in Fig. 1 indicate that both Nef73-specific and Nef84-specific CTLs were effectively elicited in chronically HIV-1-infected HLA-$A*1101^+$ individuals.

Association of an HLA-A*1101 allele with mutations in the 3 CTL epitopes. We speculated that these 2 Nef epitope-specific CTLs having a strong ability to suppress HIV-1 replication could select escape mutants but that Gag349-specific CTLs having a weak ability to suppress HIV-1 replication could not. We therefore analyzed the sequences of these epitopes and their flanking regions from HLA-$A*1101^+$ and HLA-$A*1101^-$ individuals who had been chronically infected with HIV-1 to clarify whether they selected the escape mutations. In the Gag349 epitope, only the 9S mutation was found, but there was no significant difference in the frequency of this mutation between the HLA-A*1101-positive and -negative individuals (Fig. 4A). In the Nef73 epitope, several mutations were found at positions 2, 4, 5, 8, 9, and 10 (Fig. 4B). The 9F mutation was frequently found, but there was no significant difference in the frequency of this mutation, nor in that of the other mutations, between the HLA-A*1101-positive and -negative subjects. In the Nef84 epiope, there were several mutations, at positions 2, 3, 5, 6, 7, 8, and 9, though the mutations at positions 2, 6, and 9 were the most frequently detected ones (Fig. 4C). The frequency of the Arg mutation at position 9 was significantly higher in HLA-A*1101-positive individuals than in HLA-A*1101-negative ones ($P < 0.0001$) (Fig. 4C). In contrast, the mutations at position 2 were significantly more fre-

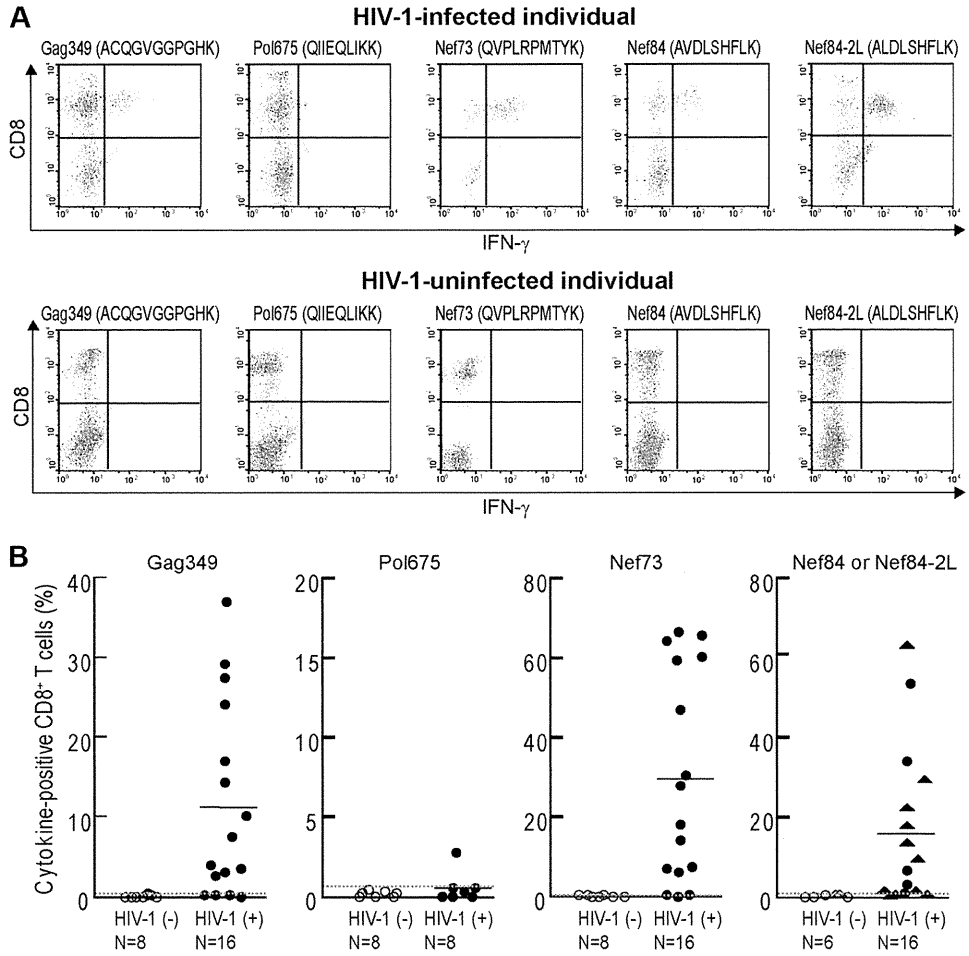

FIG. 1. Four HLA-A*1101-restricted HIV-1-specific $CD8^+$ T cells in chronically HIV-1-infected HLA-A*1101^+ individuals. (A) After PBMC from an HLA-A*1101^+ HIV-1-infected and HIV-1-uninfected individuals had been stimulated singly with each of the indicated peptides for 2 weeks, HIV-1-specific $CD8^+$ T cells were detected by measuring IFN-γ-producing $CD8^+$ T cells in the culture after stimulation with the corresponding peptide-pulsed cells. Either Nef84 or Nef84-2L peptide was used for individuals infected with HIV carrying the corresponding sequence. A representative result is shown. (B) Summary of ICC assays for HLA-A*1101^+ HIV-1-infected individuals and HIV-1-uninfected individuals. For detection of Nef84- and Nef84-2L-specific $CD8^+$ T cells, Nef84 and Nef84-2L peptides were incubated with cells from individuals infected with the wild type or the 2F and 2L viruses, respectively. The circle symbols and the triangle symbols represent the frequency of IFN-γ-producing $CD8^+$ T cells after stimulation with Nef84 and Nef84-2L peptides, respectively. The average + 3 SD of IFN-γ-producing $CD8^+$ T cells in HIV-1-uninfected individuals was defined as a positive value (Gag349, >0.34%; Pol675, >0.56%; Nef73, >0.32%; Nef84 or Nef84-2L, >0.63%). Dotted lines indicate the average + 3 SD, and solid lines indicate the average in HIV-1-infected individuals.

quently detected for HLA-A*1101-negative individuals than for HLA-A*1101-positive ones ($P = 0.045$), suggesting that they were not selected by HLA-A*1101-restricted CTLs. There were 3 mutations (Phe, Tyr, and Arg) at position 6. The frequency of each one at position 6 was not significantly higher for HLA-A*1101-positive individuals than for HLA-A*1101-negative ones. These results together suggest that only the 9R mutation was selected by Nef84-specific CTLs.

There were several mutations in the flanking region of these epitopes, but no significant difference in them between the HLA-A*1101-positive and -negative individuals was found (data not shown).

In vitro recognition of the 9R mutation by Nef84-specific CTLs. We speculated that the 9R mutant is an escape mutant from Nef84-specific CTLs because this mutation is associated with the HLA-A*1101 allele. We therefore investigated whether or not the Nef84-specific CTLs could recognize the Nef84-9R mutant epitope. We first tested the activity of Nef84-specific CTL clones in killing target cells prepulsed with the Nef84-9R mutant peptide. Three Nef84-specific CTL clones effectively killed target cells prepulsed with Nef84 or Nef84-2L wild-type peptide but failed to kill those prepulsed with Nef84-2L9R peptides (Fig. 5A). The results of an HLA class I stabilization assay showed that the affinity of the Nef84-2L9R peptide for HLA-A*1101 was much weaker than that of Nef84 or Nef84-2L for it (Fig. 5B). Taken together, these results suggest that the Nef84-2L9R peptide is very weakly presented in HIV-1 mutant virus-infected cells because of the very low affinity of Nef84-2L9R peptide for HLA-A*1101. We generated an NL-432 mutant carrying 2L and 9R mutations of Nef84 (NL-432-Nef84-2L9R) virus and infected HLA-A*1101^+ $CD4^+$ T cells with this virus. The infected cells showed down-

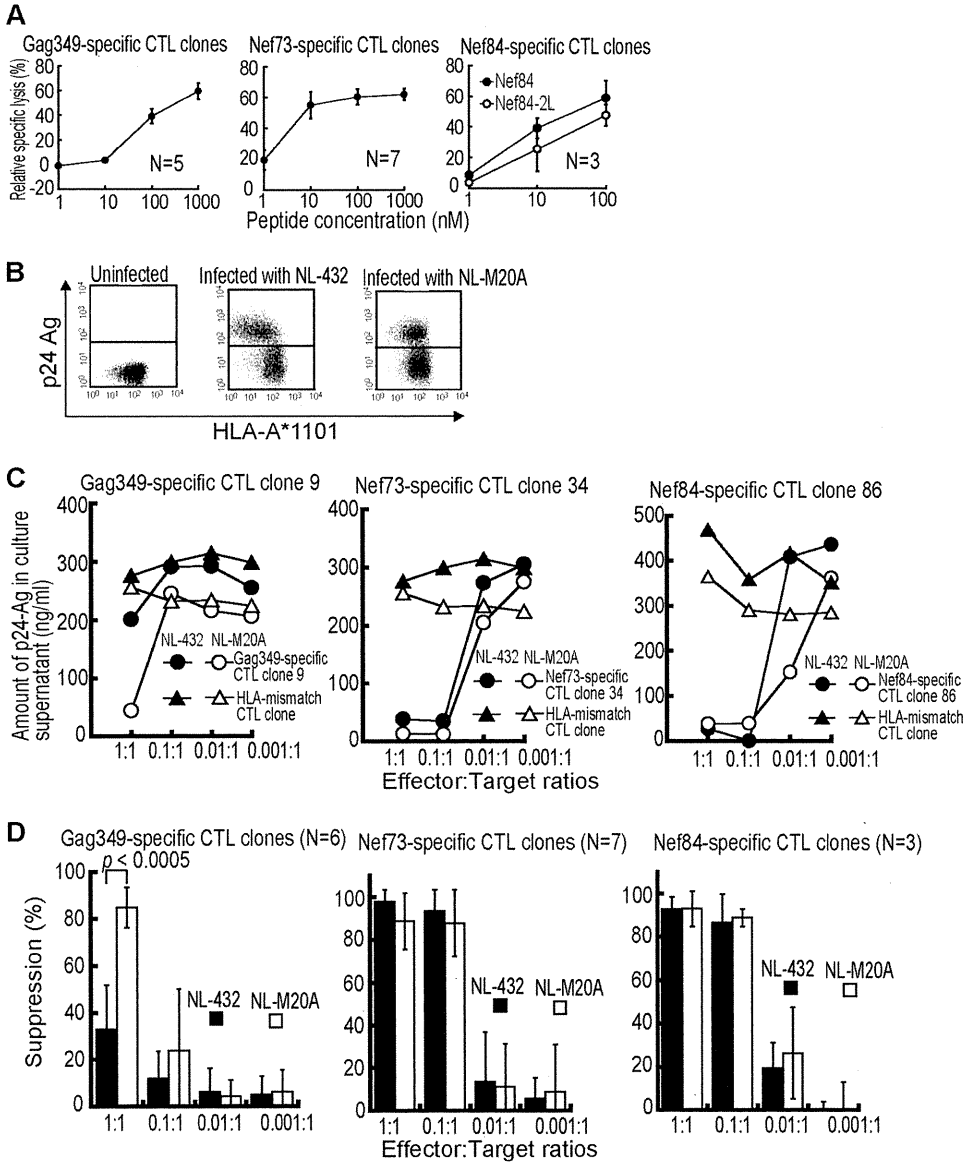

FIG. 2. Ability of HLA-A*1101-restricted CTLs to suppress HIV-1 replication in HIV-1-infected $CD4^+$ T cells. (A) Cytolytic activities of HLA-A*1101-restricted HIV-1-specific CTLs (5 Gag349-specific, 7 Nef73-specific, and 3 Nef84 consensus B-specific CTL clones) were tested by using C1R-A*1101 cells pulsed with various concentrations of the corresponding peptide (effector-to-target-cell ratio = 2:1). (B) Surface expression of HLA class I molecules on $CD4^+$ T cells infected with HIV-1 NL-432 or NL-M20A. $CD4^+$ T cells infected with HIV-1 NL-432 or NL-M20A were stained with anti-HLA-A*1101 and anti-p24 MAbs and then analyzed by using flow cytometry. (C) Ability of HLA-A*1101-restricted CTLs to suppress HIV-1 replication in cultures of HIV-1-infected $CD4^+$ T cells. $CD4^+$ T cells from an HLA-A*1101^+ healthy individual were infected with NL-432 or NL-M20A and then cocultured with HLA-A*1101-restricted CTL clones or HLA-mismatch CTL clone (HLA-B*5101) at various effector-to-target ratios. HIV-1 p24 Ags in the supernatant were measured on day 6 or 7 postinfection by conducting an enzyme immunoassay. (D) Analysis using multiple HLA-A*1101-restricted CTLs to suppress replication of NL-432 or NL-M20A.

regulation of HLA-A*1101 on target cells infected with NL-432 or NL-432-Nef84-2L9R but not on those infected with NL-M20A (Fig. 5C). Thus, these result also revealed that the 2L9R mutations do not affect the downregulation of HLA class I molecules. Three Nef84-specific CTL clones failed to suppress replication of NL-432-Nef84-2L9R (Fig. 5D), whereas these T-cell clones effectively suppressed replication of NL-432 at E:T ratios of 1:1 and 0.1:1 (Fig. 5D and 5E). These results indicate that the CTL clones could not recognize cells infected with NL-432-Nef84-2L9R and confirmed 9R to be an escape mutation.

Different surface expression levelS of PD-1 between Nef73-specific and Nef84-specific CTLs. Both Nef73-specific and Nef84-specific CTL clones effectively suppressed HIV-1 replication *in vitro*. In contrast, the latter CTLs selected an escape mutation *in vivo*, whereas the former ones did not. These findings suggest the possibility that Nef73-specific CTLs cannot mediate selection of escape mutants *in vivo*. PD-1 expression

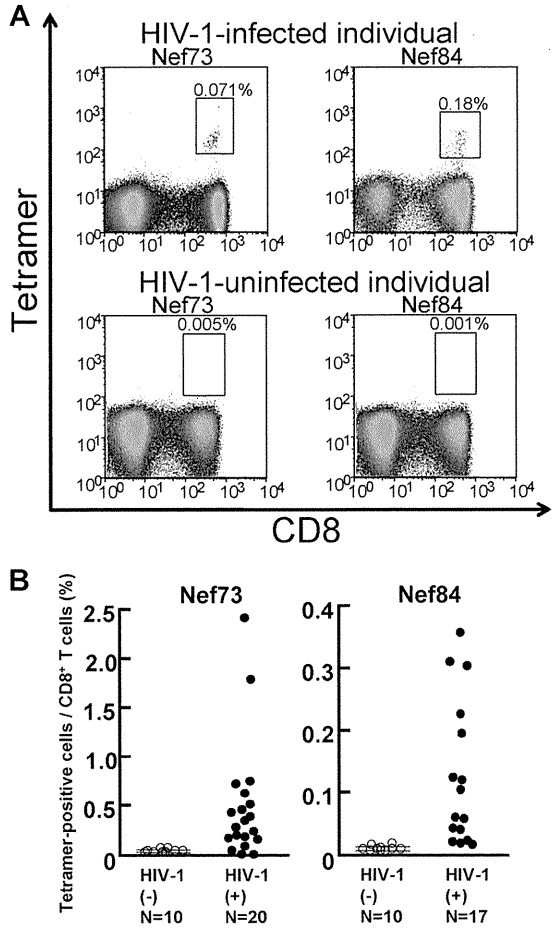

FIG. 3. Frequency of HLA-A*1101-restricted Nef epitope-specific $CD8^+$ T cells. PBMCs from HLA-A*1101^+ HIV-1-infected or HIV-1-uninfected individuals were examined by using Nef73-specific or Nef84-specific tetramers and anti-CD8 MAb or by using only anti-CD8 MAb. (A) A representative result of Nef73-specific or Nef84-specific tetramer binding $CD8^+$ T cells. (B) Summary of frequency of HLA-A*1101^+-restriced Nef73-specific or Nef84-specific $CD8^+$ T cells in HIV-1-infected individuals and HIV-1-uninfected individuals. The mean frequencies + 3 SD of Nef73-specific and Nef83-specific $CD8^+$ T cells among total $CD8^+$ T cells from the HIV-1-uninfected individuals were 0.032% + 0.045% and 0.009% + 0.012%, respectively. More than 0.077% and 0.021% were evaluated as showing positive binding of Nef73-specific and Nef84-specific tetramers, respectively.

on HIV-1-specific T cells is known to be associated with dysfunction of T cells (15, 35, 44, 47). Therefore, high expression of PD-1 on the CTL surface is a possible reason why Nef73-specific CTLs failed to select escape mutants. To clarify the PD-1 expression on Nef73-specific and Nef84-specific CTLs, we stained PBMCs from HLA-A*1101^+ HIV-1-infected individuals with anti-PD-1 and anti-CD8 MAbs aND with the specific tetramer (Fig. 6A and B). Nef73-specific and Nef84-specific $CD8^+$ T cells were, respectively, detected by the tetramers in 16 and 13 chronically HIV-1-infected individuals carrying HLA-A*1101 (Fig. 3), but only 15 and 8 individuals had a sufficient number of Nef73-specific and Nef84-specific $CD8^+$ T cells for analysis of PD-1 expression, respectively. The Nef73-specific $CD8^+$ T cells expressed a significantly higher level of PD-1 than the Nef84-specific ones (Fig. 6C). But only 8 individuals (2 having the 9R mutant and 6 having wild-type Nef84) had enough Nef84-specific $CD8^+$ T cells for analysis of PD-1 expression. We did not find any difference in the expression levels of PD-1 between these 2 groups (see Fig. S1 in the supplemental material). These results suggest that the 9R mutation did not influence the level of PD-1 on Nef84-specific $CD8^+$ T cells.

Both Nef84- and Nef73-specific CTLs, enough for the analysis of PD-1 expression, were detected in only 5 of the individuals tested. We compared the levels of PD-1 between the CTLs within the same individual. A similar difference was found between these CTLs within each individual (Fig. 6D). PD-1 is known to be upregulated on activated T cells (34). Therefore, we speculate that Nef84-specific CTLs are not activated, because the wild-type virus disappeared and the Nef84-9R escape mutant was selected in many HLA-A*1101^+ individuals, resulting in downregulation of PD-1 expression on the T cells. We investigated the sequences of these Nef epitopes in HIV-1 from the 5 individuals whose Nef73-specific and Nef84-specific $CD8^+$ T cells were analyzed for PD-1 expression. These 5 individuals were infected with HIV-1 carrying the wild-type Nef73 sequence, whereas the sequence of Nef84 was wild type (2V or 2L) in 3 of these individuals, Nef84-9R in 1, and a mixture of both in 1 individual (Table 1). The Nef84-specific $CD8^+$ T cells from the individual infected with the Nef84-9R mutant (KI-390) expressed the highest level of PD-1 among the T cells from these 5 individuals (Fig. 6D). Together with the results showing no difference in the expression levels of PD-1 between individuals infected with the 9R mutant and those infected with the wild-type virus, these results exclude the possibility that the lower level of expression of PD-1 on Nef84-specific T cells resulted from the appearance of the Nef84-9R mutant virus in these individuals.

A recent study showed that PD-1 is highly expressed on effector memory T cells and that its expression is related to the differentiation of $CD8^+$ T cells (37). Therefore, the difference in expression of PD-1 may result from the difference in differentiation status between these 2 Nef epitope-specific T cells. We analyzed the CD27 CD28 CD45RA phenotype of these T cells in the 5 individuals to clarify differentiation of the T cells. The results showed no difference in differentiation status between these 2 Nef epitope-specific T cells, although effector and late effector subsets were predominantly detected in Nef84-specific and Nef73-specific T cells from one individual (see Fig. S2 in the supplemental material). These results indicate that a difference in expression of PD-1 between these T cells was not due to the difference in differentiation status.

We speculate that there is no difference in the level of PD-1 expression between Nef73-specific and Nef84-specific CTL clones, because both CTL clones showed strong ability to suppress HIV-1 replication. To complement the *ex vivo* data, we analyzed the PD-1 expression on our *in vitro*-generated CTL clones. The results showed that both CTL clones expressed a low level of PD-1 and that there was no difference in the expression level between these CTL clones (data not shown).

We further investigated PD-1 expression on 2 CTLs having a strong ability to suppress HIV-1 replication. HLA-A*2402-restricted Nef138-specific CTLs were recently shown to have a strong ability to suppress HIV-1 replication and to select