Strong ability of Nef-specific CD4+ cytotoxic T cells to suppress HIV-1 replication in HIV-1-infected CD4+ T cells and macrophages

Nan Zheng1, Mamoru Fujiwara1, Takamasa Ueno1, Shinichi Oka2,3, and Masafumi Takiguchi1

1Division of Viral Immunology and 2Division of Infectious Disease, Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, and 3AIDS Clinical Center, International Medical Center of Japan, 1-21-1, Toyama, Shinjuku, Tokyo 162-8655, Japan

Correspondence: Masafumi Takiguchi, Division of Viral Immunology, Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan.
Tel:+81-96-373-6529, Fax:+81-96-373-6532
e-mail:masafumi@kumamoto-u.ac.jp

Running title: HIV-1-specific CD4+ CTLs

Word count: text 5,732 words, abstract 194 words
Figure/table count: 5 figures and 1 table
Abstract

A restricted number of studies have shown that 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 IFN-γ production after having been stimulated with autologous B-LCLs infected with Nef recombinant vaccinia virus or pulsed with heat-inactivated virus particles, indicating the presentation of the epitope antigen through both exogenous and endogenous MHC 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 in CD4⁺ T cells in vitro. Nef187-203-specific cytotoxic CD4⁺ T cells were detected in cultures of peptide-stimulated PBMC and in ex vivo PBMC 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.
Introduction

HIV-specific CD8+ cytotoxic T cells (CTLs) play a central role in the control of human immunodeficiency virus 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 down-regulation of MHC class 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 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 (HCMV), 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 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 PBMC 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 an HTLV-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 ability 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 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 peripheral blood mononuclear cells (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, or 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, 8 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, peptide bulk cultured PBMCs or Nef-specific CD4+ T cell clones (effector cells) were stimulated with autologous EBV-transformed B-lymphoblastoid cells (B-LCL) pre-pulsed 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% FCS before use. The mixed cells were incubated for 6 h at 37°C in 5% CO2. Brefeldin A (Sigma-Aldrich) was added at a concentration of 10 µg/ml after the first 2 hours of incubation to inhibit secretion of
cytokines. In order to determine the MHC class II restriction of the CD4+ T cell epitopes, we also employed peptide-pulsed HLA-DR allele partially matched or mismatched allogeneic B-LCL as stimulators in some assays. After a 6-hour incubation, the cells were stained with PE-conjugated anti-human CD4 mAb (BD Biosciences, San Jose, CA). Then the cells were fixed, made permeable, stained with FITC-conjugated anti-human 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 peptide bulk-cultured PBMCs or Nef-specific CD4+ T cell clones with 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, and stained with FITC-conjugated anti-human perforin, PE-conjugated anti-human granzyme A or Alexa647-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 peptide were also prepared. Cells were incubated for 6 h at 37°C in 5% CO2. Brefeldin A was added at a concentration of 10 µg/ml after the first 2 hours 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-bottomed 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 [HS] and 200U/ml recombinant human IL-2, 5 x 10⁴ irradiated allogeneic PBMC from a healthy donor as feeders, and 1 x 10⁵ irradiated autologous EBV transformed B-lymphoblastoid cells pre-pulsed with a 10⁻⁶ M concentration of the corresponding peptide). Wells positive for growth after 2-3 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% HS supplemented with 200 U of recombinant human IL-2/ml and were stimulated weekly with irradiated autologous B-LCL pre-pulsed with the appropriate epitope peptide.

**Blocking of CD4⁺ T cell responses**

To determine the MHC class II restriction of Nef-specific CD4⁺ T cell responses, we blocked the T-cell receptor-MHC class II interaction by using human MHC class 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 Dr. Nishimura. Autologous B-LCLs pre-pulsed with the Nef epitope were incubated with the appropriate antibody (10µg/ml) for 1 hour 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 hours. Brefeldin A was added to the cultures (10µg/ml) 4 hours prior to termination of the cultures. To evaluate the ability of the effector cells to produce IFN-γ under blocking, we stained the cells after stimulation with PE-conjugated anti-human CD4 mAb. Then the cells were fixed, made permeable,
Intracellular cytokine production (ICC) assays for stimulator cells infected with recombinant vaccinia virus

Autologous B-LCLs were infected with 10 plaque-forming units per cell of recombinant HIV-1 Nef vaccinia virus (rVac-Nef), or wild-type vaccinia virus (Vac-WT) and cultured for 16 h at 37°C in 5% CO₂. 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 hours. Brefeldin A was present in the cultures (10 µg/ml) for the last 4 hours. 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 minutes. Autologous B-LCLs were incubated with the inactivated virus particles at 0.5 µg/ml (p24 antigen concentration) for 16 hours at 37°C in 5% CO₂. 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 hours. Brefeldin A was present in the cultures (10 µg/ml) for the last 4 hours. To evaluate the ability of effector cells to produce IFN-γ the cells 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-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 x 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 1.5-3 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 peripheral blood mononuclear cells (PBMC) of a HLA-DRB1*0803-positive or HLA-DRB1*0403-positive healthy donor by using anti–human CD14 monoclonal antibody (mAb)–coated and anti–human CD4 mAb–coated magnetic beads (magnetic-activated cell sorting [MACS] beads, Miltenyi Biotec, Bergisch Galdbach, Germany), respectively. The isolated monocytes were cultured in complete medium containing M-CSF (50 ng/ml) for 1 week before use. The isolated CD4^+ T cells were cultured for 1 week in complete medium containing IL-2 (200U/ml) and IL-4 (2.5ng/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 frame shift at a XhoI site (44th amino acid of Nef protein), were kindly donated by Dr. Koyanagi (Kyoto University, Kyoto, Japan). The infectious proviral clone of pJRFLNL-432Nef was previously constructed by exchanging the Nef region of an R5 strain JRFL with that of NL-432 (17).

**CTL assay**

The cytotoxicity of Nef-specific CD4+ T cell clones against B-LCL or HIV-1-infected target cells was measured by a standard $^{51}$Cr release assay as previously described (17). Briefly, target cells (2 x $10^5$) were incubated for 60 min with 100 µCi of Na$_2$CrO$_4$ in saline and washed 3 times with RPMI 1640 medium containing 10% NCS. Labeled target cells (2 x $10^3$/well) were seeded in a 96-well round-bottomed microtiter plate (Nunc). For the assays of B-LCL, the desired amount of the corresponding peptide was co-incubated with labeled target cells for 1 hour. 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 following equation: 

$$\text{Specific lysis} = \left( \frac{\text{cpm exp} - \text{cpm spn}}{\text{cpm max} - \text{cpm spn}} \right) \times 100 \%,$$

where cpm exp is the counts per minute in the supernatant in the wells containing both target and effector.
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 hours at 37°C in 5% CO₂. After 2 washes with RPMI 1640-10% FCS, the cells were co-cultured with the CD4⁺ CTL clones. From day 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 [ELISA] 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 CD4⁺ T cells cultured with HIV-1-specific CTLs / concentration of p24 Ag in the supernatant of HIV-1-infected CD4⁺ T cells culture without the CTLs) × 100.
Results

Identification and characterization of 2 HIV-1 Nef-specific CD4+ T cell epitopes

PBMCs from 2 HIV-1-seropositive individuals, KI-010 and KI-197, were cultured for 14 days after the stimulation of either of 4 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 lymphoblastoid cells (B-LCLs) pre-pulsed 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-LCL pulsed with each peptide included in the corresponding peptide cocktails. Nef17-7 and Nef17-8 peptides induced specific CD4+ T cell responses by the PBMC 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 class II-binding peptides, the core binding-region is usually not the optimal ligand for MHC class 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 2 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 the stimulation with the peptide-pulsed autologous B-LCLs, whereas HLA-DQ-specific mAbs Hu11 or Hu18 and HLA-DP-specific mAb B7/21 failed to block it (Fig. 1C). These results indicate that these 2 epitope-specific T cell responses were restricted by HLA-DR. To determine the exact restriction alleles, we stimulated Nef37-53-specific and Nef187-203-specific CD4+ T cell clones with peptide-prepulsed B-LCLs from HLA-DR partially matched or mismatched allogenors. The Nef37-53-specific CD4+ T cell clone produced IFN-γ after the stimulation with the corresponding peptide-pulsed B-LCLs from the donors sharing DRB1*0403, while the Nef187-203-specific clone produced IFN-γ after the 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 HIV-1 Nef recombinant vaccinia-infected or HIV-1 Nef protein-pulsed cells

To clarify the natural occurring presentation of these 2 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 Nef recombinant vaccinia virus (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 ability to produce IFN-γ (>95%) after the stimulation with peptide-pulsed autologous B-LCL (Fig. 2A). The B-LCLs infected with rVac-Nef induced about 70% of the 2 Nef-specific CD4+ T cell clones to produce IFN-γ, whereas those cells infected with wild-type vaccinia virus (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 301 (Nef-depleted) virus particles failed to induce IFN-γ-production (Fig. 2C). This result 302 suggests that the Nef-specific CD4+ T cells also recognized the epitope antigen presented 303 through endogenous MHC class II processing pathways. However, it still remains possible 304 that Nef proteins from cells expressing Nef killed by vaccinia or HIV infection were 305 presented by the exogenous HLA class II pathway. To exclude this possibility, we used 306 stimulator cells transfected with Nef-GFP mRNA. Nef-GFP mRNA-transfected autologous 307 B-LCLs induced IFN-γ-production from both Nef37-53-specific and Nef187-203-specific 308 CD4+ T cell clones, whereas GFP mRNA-transfected cells did not (Fig. 2D). In this assay, 309 B-LCLs were used as stimulator cells within 3 h after the transfection. The frequency of 310 dead cells among the Nef+ cells was approximately 0.6 %. These results support the idea 311 that endogenous HIV-1 Nef can be processed to MHC class II molecules similarly as in the 312 previous observation of endogenous presentation of HCMV CD4+ CTL epitopes (20). 313 Thus, our results indicate that the Nef-specific CD4+ T cells recognized the epitope antigen 314 presented through both exogenous and endogenous MHC class II processing pathways.

Cytotoxic activity and cytotoxic effector molecule expression of HIV-1 Nef-specific 317 CD4+ T cells

Although antigen-specific CD4+ T cells are classically thought to function as helper T 319 cells in antiviral immunity, HIV-1 Gag-specific cytotoxic CD4+ T cells were previously 320 reported to exist (30-32, 50). In our study, Nef37-53-specific and Nef187-203-specific 321 CD4+ T cell clones were tested for their ability to lyse autologous B-LCLs incubated with 322 the epitope peptide (1000 nM) at an E/T ratio of 5:1 (Fig. 3A). The Nef187-203-specific 323 CD4+ T cell clone showed a strong lytic activity toward autologous B-LCLs incubated with 324 the peptide, whereas the Nef37-53-specific CD4+ T cell clone did not lyse autologous
Furthermore, we stained for 3 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 both clones showed a similar level 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 cytotoxic activity of these 2 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 is suspected to exist among the PBMCs of these patients; and thus these cells would fail to kill the target cells in a chromium release assay. Epitope-specific CD4+ T cells among the PBMCs from 2 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 hours (Fig. 3C). We then gated the IFN-γ-producing CD4+ T cells and compared the cell-surface expression of CD107 between these 2 CD4+ T cells. The results showed that about 50% of Nef187-203-specific CD4+ T cells expressed CD107a on their cell surface, 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 towards HIV-1-infected macrophages and CD4+ T cells. To exclude the probability that different Nef sequences between 2 HIV-1 strains, NL-432 and JRFL, would affect the recognition of Nef-specific CD4+ CTLs, a chimera R5
virus, we used JRFLNL-432Nef, 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 JRFLNL-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 cells at day 3 post infection, 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 toward autologous B-LCLs incubated with 1000 nM peptide, which lysis was dramatically decreased when the B-LCL 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 in the case 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 towards infected macrophages was higher than that towards the infected CD4\(^+\) T cells. This difference may result from the intracellular p24 antigen expression levels between these 2 targets used in this assay (Fig. 4A).

**Ability of HIV-1 Nef-specific cytotoxic CD4\(^+\) T cells to suppress HIV-1 replication in CD4\(^+\) T cells**

A previous study showed that Gag-specific CD4\(^+\) CTL can suppress HIV-1 replication in an HTLV-I-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 the 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-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 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 toward 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 stronger ability to suppress HIV-1 replication and that non-cytotoxic 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 9 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-\(\gamma\)-producing cells were determined by intracellular staining after restimulation of the bulk cultures with HLA-DRB1*0803-positive B-LCL pre-pulsed with the peptide. We observed Nef187-203-specific CD4\(^+\) T cells in the bulk cultures from 3 of these 9 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 \textit{in vitro} cultured PBMCs, we also detected Nef187-203-specific CD4\(^+\) T cells directly \textit{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 2 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 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 2 Nef CD4+ T cell epitopes, Nef37-53 and Nef187-203, from 2 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 class II restriction of it was not reported (27). Here we characterized both 2 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 were 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 class II molecules through the endogenous pathway during an HIV-1 infection. Thus, we demonstrated for the first time both endogenous and exogenous presentations 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 non-cytotoxic 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 reality of 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 2 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, making a 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 only replicate in activated CD4+ T cells, which accomplish MHC class II expression and susceptibility 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 towards 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 that 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 down-regulation 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 in cytotoxic activity towards HIV-1-infected macrophages and 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 2 ways, by suppressing cytotoxic activity or cytokine production. A recent study showed that in vitro cultured non-cytotoxic 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+ CTL 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 non-lymphoid tissues such as lung or brain (43). Therefore, the strong ability of Nef-specific CD4+ CTL to suppress HIV-1 replication in macrophages might help to control HIV-1 rebound in STI (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 class 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. (32), this effect may result in depletion of healthy immune cells during an HIV infection. Taken together, 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 MHC class II tetramers (21).

Overall, our results demonstrated that Nef-specific cytotoxic CD4$^+$ T cells killed HIV-1-infected CD4$^+$ T cells and macrophages through perforin-mediated killing 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.
Acknowledgements

The authors thank Sachiko Sakai for secretarial assistance.

This research was supported by the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases and by the Global COE program “Global Education and Research Center Aiming at the control of AIDS” supported by the Ministry of Education, Science, Sports and Culture, Japan; by a grant-in-aid (No. 20390134) for scientific research from the Ministry of Health, Japan; by a grant-in-aid (No. 18390141) for scientific research from the Ministry of Education, Science, Sports and Culture, Japan; and by a grant from the Japan Health Science Foundation.
References


dengue virus NS1-NS2a proteins by human CD4+ cytotoxic T lymphocyte clones.


Figure Legends

Figure 1. Identification and characterization of 2 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 2 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-LCL 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 pre-pulsed 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 hour. Then the 2 Nef epitope-specific CD4+ T cell clones were stimulated with the MHC II-specific antibody-treated B-LCLs at an effector-to-stimulator 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, HLA-DR partially matched and mismatched allogeneic 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.

Figure 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-infected, HIV-1 virus 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 pre-pulsed 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 recombinant Nef vaccinia virus (rVac-Nef) or wild-type vaccinia virus (Vac-WT). (C) Nef-specific CD4+ T cell clones were tested for their IFN-γ production after stimulation with B-LCLs pre-pulsed with heat-inactivated HIV-1 virus particles of an 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.

Figure 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 towards autologous B-LCLs incubated with the epitope peptides (1000 nM) were measured by a standard 51Cr release assay at an effector-to-target (E/T) ratio of 5:1. Bar designations: 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 frequency (%) of each subset 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, same clones stained with isotype control antibody. Values in histograms show mean fluorescence intensity (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 2 HIV-1-seropositive donors, KI-010 and KI-197, were incubated with/or without their corresponding epitope peptide for 6 hours. Then these PBMCs were stained with anti-CD4, anti-IFN-γ, and anti-CD107a or with mouse IgG mAb as an isotype control. Values in the IFN-γ/CD4 dot plots indicate the frequency 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/SSC dot plots indicate the frequency of the high-fluorescent subsets in the gated CD4+IFN-γ+ population of the PBMCs stained with PE-conjugated anti-CD107a (CD107a-PE) or of the same PBMCs stained with PE-conjugated mouse IgG isotype mAb (Isotype-PE).

**Figure 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 a HLA-DRB1*0803-positive donor at day 3 post infection. The dashed histogram represents...
uninfected cells; and the bold histogram, HIV-1-infected cells. The values in each plot show
the frequency of p24 antigen-positive cells. The uninfected and HIV-1-infected
macrophages and CD4+ T cells were then labeled with Na$_2$CrO$_4$ and incubated with
Nef187-203-specific clones for CTL assays. (B) Cytotoxic activity of 3
Nef187-203-specific clones towards autologous B-LCL incubated with the peptide at the
indicated concentrations. The cells were tested at an E/T ratio of 5:1. (C) Ability of
Nef187-203-specific clones to lyse HIV-1-infected or uninfected macrophages 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 3 Nef187-203-specific clones.

Figure 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$_{NL-432Nef}$ 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 the HIV-1
and subsequently co-cultured or not with Nef-187-203-specific CD4+ CTL clones at an E/T
ratio of 0.1:1. The concentration of p24 antigen in the supernatant on day 6 post infection
was measured by using an enzyme immunoassay. Values are presented as the averages ±
standard deviations of results from the assays of 2 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$_{NL-432Nef}$-infected macrophages or NL-432-infected CD4+ T
cells were subsequently co-cultured with a Nef187-203-specific clone at the indicated E/T
ratios. The concentration of p24 antigen in the supernatant on day 6 post infection 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 2 healthy donors expressing the corresponding HLA-DR alleles were infected with HIV-1, and were subsequently co-cultured 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 post infection 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 lower than that ability of Nef187-203-specific CD4+ CTL clones. Values are present as averages ± standard deviations (error bars) of results from the assays of 3 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.
Table 1. Detection of Nef187-203-specific CD4⁺ T cells in chronically HIV-1-infected individuals

<table>
<thead>
<tr>
<th>Subject</th>
<th>HAART</th>
<th>CD4 count (cells/ml)</th>
<th>Viral load (RNA copies/ml)</th>
<th>Cultured PBMCs</th>
<th>Ex vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% CD4⁺ IFN-γ⁺</td>
<td>% CD4⁺ IFN-γ⁺</td>
<td>% CD4⁺ IFN-γ⁺ CD107a⁺</td>
<td></td>
</tr>
<tr>
<td>KI-097</td>
<td>+</td>
<td>322</td>
<td>14,000</td>
<td>0</td>
<td>nt</td>
</tr>
<tr>
<td>KI-105</td>
<td>+</td>
<td>485</td>
<td>&lt;50</td>
<td>2.2</td>
<td>0</td>
</tr>
<tr>
<td>KI-121</td>
<td>-</td>
<td>265</td>
<td>24,000</td>
<td>18.4</td>
<td>0</td>
</tr>
<tr>
<td>KI-139</td>
<td>+</td>
<td>505</td>
<td>110,000</td>
<td>0</td>
<td>nt</td>
</tr>
<tr>
<td>KI-144</td>
<td>+</td>
<td>496</td>
<td>17,000</td>
<td>0</td>
<td>nt</td>
</tr>
<tr>
<td>KI-152</td>
<td>+</td>
<td>303</td>
<td>&lt;50</td>
<td>0</td>
<td>nt</td>
</tr>
<tr>
<td>KI-154</td>
<td>+</td>
<td>481</td>
<td>7,700</td>
<td>70.3</td>
<td>0.01</td>
</tr>
<tr>
<td>KI-163</td>
<td>+</td>
<td>419</td>
<td>26,000</td>
<td>0</td>
<td>nt</td>
</tr>
<tr>
<td>KI-185</td>
<td>+</td>
<td>331</td>
<td>&lt;50</td>
<td>0</td>
<td>nt</td>
</tr>
<tr>
<td>KI-197</td>
<td>+</td>
<td>350</td>
<td>&lt;50</td>
<td>60.7</td>
<td>0.06</td>
</tr>
</tbody>
</table>

nt: not tested

% CD4⁺ IFN-γ⁺ is the frequency of IFN-γ⁺-producing cells among CD4⁺ cells. % CD4⁺ IFN-γ⁺ CD107a⁺ is the frequency of CD107a⁺ cells among Nef187-203-specific CD4⁺IFN-γ⁺ cells.