*3, *4, and 1.8-kb species) among 5R/low expressors and high expressors. Since these transcripts do not contain the RRE region, it is clear that they are generated via splicing downstream of SA1. The abundance of these transcripts (*3, *4, and 1.8-kb species) for 5R and low expressors may also imply that the overall splicing efficiency of these clones is higher than that of high expressors. Efficient splicing at SA sites may compete with Rev function, and equilibrium between the strength of splicing acceptors and Rev function for the nuclear export of Rev-dependent mRNAs is important for virus replication (28). Thus, increased splicing for 5R and low expressors may obstruct the function of Rev, which results in a decrease in the Rev-dependent expression of late proteins from RRE-containing transcripts. On the other hand, while the amounts of 1.8-kb mRNAs of 5R and low expressors were larger than those of high expressors, the expression levels of viral early proteins were similar among 5R and its variants. A high concentration of Rev was previously shown to inhibit the translation from various RNAs (47). It is possible that the expression of viral early proteins may be regulated at an optimal level for viral replication. Alternatively, the translation efficiency of ~40 mRNA isoforms synthesized by alternative splicing events may vary due to differences in their noncoding sequences and/or structures. Viral mRNA species within 1.8-kb and 4-kb RNAs were shown to be altered by mutations that change splicing efficiency at SA1 or the structure of SLSA1 (27, 45, 48). Viral mRNA isoforms with a low translation efficiency, even if present in abundance, may not express a high level of their corresponding proteins.

vif mRNA expression is strongly influenced by splicing efficiency at the SA1 site. The regulation of splicing at SA1 is complicated and is determined by various elements, including three different exonic splicing enhancers (ESE-Vif and ESE-M1 [Fig. 9A] and ESE-M2 [nt 4956 to 4962 in NL4-3]), a suboptimal D2 splicing site (nt 4960 to 4970 in NL4-3), a GGGG silencer (nt 4968 to 4971 in NL4-3), and a G run (G_{12}-1, nt 5034 to 5038 in NL4-3), which are located within the region from SA1 to just upstream of the *vif* start codon (nt 5041 in NL4-3) (27, 28, 48). The proviral clone 5R was constructed by introducing SIVmac239 *vif* into the downstream region of the *pol* open reading frame in the NL4-3 genome (Fig. 1 and 9B) (34). As a result, while the *pol* and *vif* genes of NL4-3 partially overlap, those of 5R do not. Since splicing efficiency is dependent on the sequence around the splice sites and their distance from the regulatory elements, the insertion of SIVmac239 *vif* into NL4-3 may have changed the splicing event at SA1. Indeed, 5R produced abundant amounts of the *vif* transcript (the *1 species in Fig. 9). The increase in *vif* mRNA was previously shown to decrease virion production, and the proportion between unspliced and spliced mRNAs has been suggested to be important for virion production (27). In agreement with this finding, we found that the virion production level from 293T cells transfected with 5R was lower than that from cells transfected with NL4-3 (data not shown). The decrease in *vif* transcript (*1 species) expression for high expressors may have caused the increase in virion production.

The splicing balance of viral mRNAs has been suggested to have biologically significant effects on viral replication (4, 9–11). Accumulating evidence has shown that HIV-1 gene expression processes, composed of transcription, poly(A) tailing, splicing, mRNA export, and subsequent translation, are mutually affected and coupled, even though these processes are biochemically distinguished (1, 2, 49). In addition, various elements within the HIV-1 genome and a number of virus/host factors have been shown to be involved in HIV-1 gene expression (3, 4, 9–11, 25–30, 50–55). The virological importance of the nucleotide sequence in the SA1prox is evident from the increase or decrease in viral replication caused by naturally occurring single-nucleotide changes. Further studies are needed to elucidate the molecular mechanism underlying the modulation of overall HIV-1 gene expression generated by single-nucleotide changes in the SA1prox.

ACKNOWLEDGMENTS

This study was supported in part by a grant from the Ministry of Health, Labor and Welfare of Japan (Research on HIV/AIDS project no. H24-005).

We are indebted to the NIH AIDS Research and Reference Reagent Program and Immuno Ltd./the MRC AIDS Directed Programme Reagent Project for antibodies. We thank Kazuko Yoshida for her editorial assistance.

We declare that no competing interests exist.

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Vaccine-Induced CD107a$^+$ CD4$^+$ T Cells Are Resistant to Depletion following AIDS Virus Infection

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ABSTRACT

CD4$^+$ T-cell responses are crucial for effective antibody and CD8$^+$ T-cell induction following virus infection. However, virus-specific CD4$^+$ T cells can be preferential targets for human immunodeficiency virus (HIV) infection. HIV-specific CD4$^+$ T-cell induction by vaccination may thus result in enhancement of virus replication following infection. In the present study, we show that vaccine-elicited CD4$^+$ T cells expressing CD107a are relatively resistant to depletion in a macaque AIDS model. Comparison of virus-specific CD107a, macrophage inflammatory protein-1β, gamma interferon, tumor necrosis factor alpha, and interleukin-2 responses in CD4$^+$ T cells of vaccinated macaques prechallenge and 1 week postchallenge showed a significant reduction in the CD107a$^-$ but not the CD107a$^+$ subset after virus exposure. Those vaccinees that failed to control viremia showed a more marked reduction and exhibited significantly higher viral loads at week 1 than unvaccinated animals. Our results indicate that vaccine-induced CD107a$^-$ CD4$^+$ T cells are depleted following virus infection, suggesting a rationale for avoiding virus-specific CD107a$^-$ CD4$^+$ T-cell induction in HIV vaccine design.

IMPORTANCE

Induction of effective antibody and/or CD8$^+$ T-cell responses is a principal vaccine strategy against human immunodeficiency virus (HIV) infection. CD4$^+$ T-cell responses are crucial for effective antibody and CD8$^+$ T-cell induction. However, virus-specific CD4$^+$ T cells can be preferential targets for HIV infection. Here, we show that vaccine-induced virus-specific CD107a$^-$ CD4$^+$ T cells are largely depleted following infection in a macaque AIDS model. While CD4$^+$ T-cell responses are important in viral control, our results indicate that virus-specific CD107a$^-$ CD4$^+$ T-cell induction by vaccination may not lead to efficient CD4$^+$ T-cell responses following infection but rather be detrimental and accelerate viral replication in the acute phase. This suggests that HIV vaccine design should avoid virus-specific CD107a$^-$ CD4$^+$ T-cell induction. Conversely, this study found that vaccine-induced CD107a$^+$ CD4$^+$ T cells are relatively resistant to depletion following virus challenge, implying that induction of these cells may be an alternative approach toward HIV control.

Virus-specific CD8$^+$ T-cell responses play a central role in the control of human immunodeficiency virus (HIV) replication (1–6). CD8$^+$ T cells, via their T-cell receptor, specifically recognize viral epitopes bound to human leukocyte antigen (HLA) class I molecules on the surface of virus-infected cells. Previous studies on HIV-infected individuals have shown an association of several HLA genotypes with delayed AIDS progression, implying possible HIV control by effective CD8$^+$ T-cell responses (7–10). Current vaccine trials in macaque AIDS models with simian immunodeficiency virus (SIV) infection have shown that induction of effective CD8$^+$ T-cell responses can result in reduction of postchallenge viral loads (11–16). Furthermore, cumulative studies have shown protection of SIV challenge by passive immunization with neutralizing antibody in macaques, suggesting the possibility of HIV protection by vaccine-induced effective antibodies (17–19).

Virus-specific CD4$^+$ T-cell responses are crucial for induction of effective CD8$^+$ T-cell and antibody responses (20–28). CD4$^+$ T cells, however, are targets for HIV, which can be an obstacle to potent virus-specific CD4$^+$ T-cell responses following HIV infection (29–31). Because HIV preferentially infects HIV-specific CD4$^+$ T cells, induction of HIV-specific memory CD4$^+$ T cells by vaccination may increase the target cell pool for HIV infection and thus enhance viral replication (32).

Our previous trial of a prophylactic vaccine regimen of a DNA prime and a boost with a Sendai virus (SeV) vector expressing SIV Gag (SeV-Gag) showed control of an SIV challenge in some vaccinated rhesus macaques (11). Vaccine-induced Gag-specific CD8$^+$ T cells were shown to be responsible for this SIV control (33, 34). However, the effect of SIV-specific CD4$^+$ T-cell induction by vaccination on postchallenge virus replication remains unclear. Virus-specific CD4$^+$ T cells can be divided into multiple subsets producing a variety of cytokines following viral antigen stimulation (35, 36). In the present study, we examined changes in multiple subsets of vaccine-induced CD4$^+$ T cells following SIV infection in a macaque AIDS model. Comparison of SIV-specific CD4$^+$ T-cell profiles pre- and postchallenge indicated that vaccine-elicited CD4$^+$ T cells expressing CD107a are relatively resistant to depletion whereas virus-

Received 14 July 2014 Accepted 24 September 2014
Published ahead of print 1 October 2014
Editor: G. Silvestri
Address correspondence to Tetsuro Matano, tmatano@nih.go.jp.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/JVI.02032-14.

doi:10.1128/JVI.02032-14

specific CD107a$^-$ CD4$^+$ T cells are largely depleted in the postchallenge acute phase of infection. These results imply that induction of the latter CD4$^+$ T-cell subset by vaccination may result in enhanced HIV replication after virus exposure.

MATERIALS AND METHODS

Samples. The present study used frozen peripheral blood mononuclear cell (PBMC) samples derived from 18 vaccinated and 21 unvaccinated Burmese rhesus macaques (*Macaca mulatta*) for analysis of SIV-specific CD4$^+$ T-cell responses. Our previous SIVmac239 challenge experiments using these animals (34, 37–40) were conducted at the Tsukuba Primate Research Center, National Institute of Biomedical Innovation (NIBP), and the Institute for Virus Research, Kyoto University (IVRKU), with the help of the Corporation for Production and Research of Laboratory Primates. This study was approved by the Committee on the Ethics of Animal Experiments of NIBP and IVRKU under the guidelines for animal experiments at NIBP, IVRKU, and the National Institute of Infectious Diseases, which is in accordance with the Guidelines for Proper Conduct of Animal Experiments established by the Science Council of Japan (http://www.scj.go.jp/ja/info/kohyo/pdf/kohyo-20-k16-2e.pdf).

Vaccinated animals received a DNA prime and an SeV-Gag boost. The DNA used for the vaccination, CMV-SHIVdEN DNA (11), was constructed from an *env*- and *nef*-deleted simian-human immunodeficiency virus (SHIV) molecular clone DNA (SIVGP1) and has the genes encoding SIVmac239 Gag, Pol, Vif, Vpx, and a part of Vpr and HIV Tat and Rev. Animals received 5 mg of CMV-SHIVdEN DNA intramuscularly. Six weeks after the DNA prime, animals received a single boost intranasally with 1×10^8 cell infectious units (CIU) of replication-competent SeV-Gag (macaques R02-003, R02-012, R02-005, and R02-001) or 6×10^9 CIU of replication-defective F-deleted SeV-Gag ($n = 14$) (11, 41). There were no differences observed for CD4$^+$ T-cell markers between animals receiving replication-competent boosts and those receiving replication-defective boosts. Vaccinated (3 months postboost) and unvaccinated animals were intravenously challenged with 1,000 50% tissue culture infective doses ($TCID_{50}$) of SIVmac239 (42). In our previous study (34, 38), the geometric mean of viral loads at 6 months was approximately 2.5×10^5 copies/ml and the "M $-$ 2 $\times$ SD" value (where M is the mean and SD is the standard deviation) of log-transformed viral loads was 3.2 (corresponding to 1.6×10^3 copies/ml) in unvaccinated animals possessing major histocompatibility complex class I (MHC-I) haplotype *90-120-Ie*, which exhibit a typical course of SIV infection in Burmese rhesus macaques. Animals whose viral load at 6 months was less than 1.6×10^3 copies/ml were considered SIV controllers. The 21 unvaccinated animals included 17 with persistent viremia and 4 with undetectable or marginal levels of set-point plasma viral loads (see Fig. S1 in the supplemental material).

Analysis of SIV-specific CD4$^+$ T-cell responses. We examined SIV-specific induction of CD107a, macrophage inflammatory protein-1β (MIP-1β), gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), and interleukin-2 (IL-2) in CD4$^+$ T cells as described previously (38, 43, 44). In brief, 5×10^5 PBMCs were prestimulated with 5 μg/ml immobilized anti-human CD28 (BD) and 5 μg/ml immobilized anti-human CD49d (Biolegend) in 96-well U-bottom plates at 37°C for 12 h, followed by coculture at 37°C for 6 h in the presence of Alexa Fluor 647-conjugated anti-human CD107a (Biolegend) with 1×10^5 autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines (B-LCLs) infected with vesicular stomatitis virus G protein (VSV-G)-pseudotyped SIVGP1 for SIV-specific stimulation or mock B-LCLs for nonspecific stimulation. Monensin (final concentration, 0.7 μg/ml; BD) and brefeldin A (final concentration, 10 μg/ml; Sigma-Aldrich) were added to the culture 1 h after the start of coculture. The pseudotyped virus was obtained by cotransfection of 293T cells with a vesicular stomatitis virus G protein expression plasmid and an SIVGP1 DNA. SIV Gag capsid p27-positive cells detected by immunostaining were 5 to 10% of B-LCLs infected with VSV-G-pseudotyped SIVGP1. Immunostaining was performed using the Fix & Perm fixation and permeabilization kit (Invitrogen) and the following monoclonal antibodies: APC-Cy7-conjugated anti-nonhuman primate CD3 (BD), phycoerythrin (PE)-Texas Red-conjugated anti-human CD4 (Invitrogen), Alexa Fluor 700-conjugated anti-human CD8 (BD), PE-Cy7-conjugated anti-human IFN-γ (eBioscience), Pacific Blue-conjugated anti-human TNF-α (Biolegend), peridinin chlorophyll protein (PerCP)-Cy5.5-conjugated anti-human IL-2 (Biolegend), and PE-conjugated anti-human MIP-1β (BD). Dead cells were stained using the Live/Dead Fixable Dead Cell stain kit (Invitrogen).

Flow cytometric analysis was performed using FlowJo. Each subset positive for the marker of interest was determined in the dot plot gated by CD4$^+$ T cells as shown in Fig. S2 in the supplemental material. The frequency of each subset of SIV-specific CD4$^+$ T cells was calculated by subtracting the frequency after nonspecific stimulation from that after SIV-specific stimulation. As negative controls, we examined SIV-specific CD107a$^+$, MIP-1β$^+$, IFN-γ$^+$, TNF-α$^+$, and IL-2$^+$ CD4$^+$ T-cell frequencies in naive PBMCs derived from vaccinated (preprime; $n = 13$) and unvaccinated (prechallenge; $n = 16$) animals. The "M + 2 $\times$ SD" values of these negative controls, 0.031%, 0.034%, 0.028%, 0.017%, and 0.010%, were considered cutoff values for SIV-specific CD107a$^+$, MIP-1β$^+$, IFN-γ$^+$, TNF-α$^+$, and IL-2$^+$ CD4$^+$ T-cell frequencies, respectively. SIV-specific CD4$^+$ T-cell frequencies less than 0.01% are shown as 0.01% in the figures, while statistical analyses were performed by using data in which values below the cutoff were set as zero. SIV-specific CD107a$^-$ CD4$^+$ T-cell frequencies, shown in Fig. 1C, were calculated as the sum of the frequencies of CD107a$^-$ MIP-1β$^+$, IFN-γ$^+$, TNF-α$^+$, or IL-2$^+$ CD4$^+$ T cells determined by Boolean gating. In our previous analyses (41, 45), SIV-specific IFN-γ$^+$ CD4$^+$ T-cell frequencies peaked 1 week after SeV-Gag boost and were largely reduced 1 week after the peak, followed by only a gradual, <2-fold decrease for a few months until challenge. In this study, SIV-specific stimulation was performed by coculture with the E/T (effector [PBMCs]/target [B-LCLs infected with VSV-G-pseudotyped SIVGP1]) ratio of 5:1, while stimulation with the E/T ratio of 2.5:1 was confirmed to induce similar levels of responses, implying that the E/T ratio of 5:1 is sufficient for the stimulation.

Statistical analysis. Differences in two sets of measurements were examined by the Wilcoxon signed-rank test or the Mann-Whitney U test. Multiple comparisons of measurements were performed by Friedman's test and Wilcoxon signed-rank test with Bonferroni's multiple-comparison procedure or the Kruskal-Wallis test and Mann-Whitney U test with Bonferroni's multiple-comparison procedure. Correlation between T-cell frequencies and viral loads was analyzed by the Spearman's test. We set significance levels of all statistical tests at P values of <0.05.

RESULTS

SIV-specific CD4$^+$ T-cell responses pre- and postchallenge in vaccinated macaques. In the present study, we analyzed SIV-specific T-cell responses using frozen PBMC samples derived from 18 vaccinated and 21 unvaccinated Burmese rhesus macaques (see Fig. S1 in the supplemental material). These animals had been used in our previous SIVmac239 challenge experiments (34, 37–40). Vaccinated animals received a DNA prime and an SeV-Gag boost, followed by an SIVmac239 challenge at 3 months postboost. Eleven vaccinated animals, referred to as vaccinated controllers (v-C), showed undetectable or marginal levels of set-point plasma viral loads, whereas the remaining seven, referred to as vaccinated noncontrollers (v-NC), failed to control SIV replication (see Fig. S1 in the supplemental material).

We examined SIV-specific CD4$^+$ T-cell responses by measurement of five markers, CD107a, MIP-1β, IFN-γ, TNF-α, and IL-2, after SIV-specific stimulation (35, 36, 38, 44). We used an *env*- and *nef*-deleted SHIV molecular clone DNA, SIVGP1, to measure the frequencies of T cells responding to SIVGP1-transduced cells (referred to as SIV-specific T cells) (11, 33). The DNA used for the prime and SIVGP1 both encode SIVmac239 Gag, Pol, Vif, Vpx,

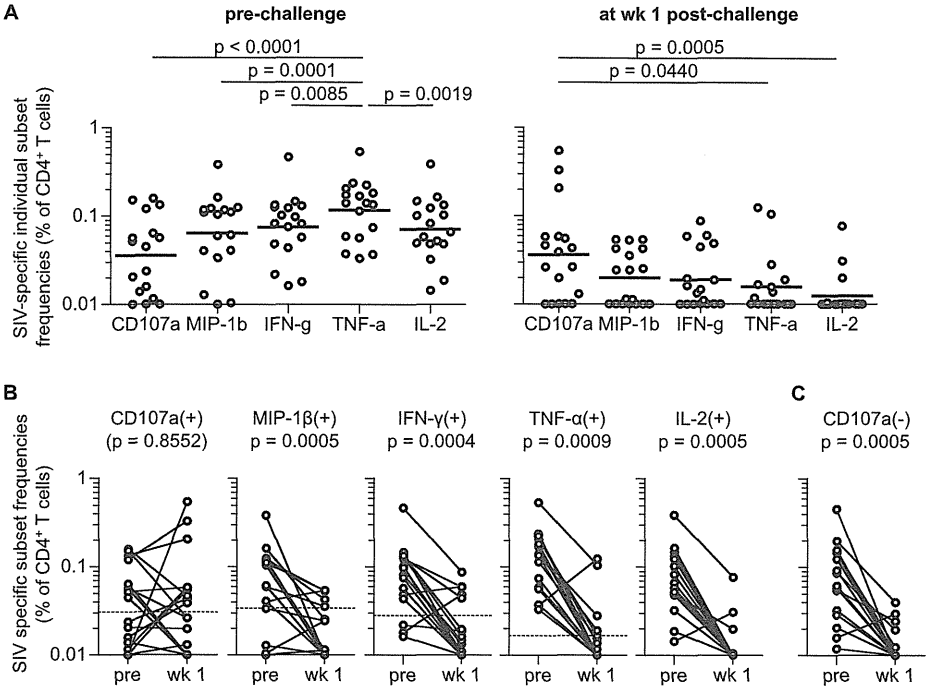

FIG 1 SIV-specific $CD4^+$ T-cell responses before and after SIV challenge in vaccinated macaques. (A) SIV-specific $CD107a^+$, MIP-$1\beta^+$, IFN-γ^+, TNF-α^+, and IL-2^+ frequencies in total $CD4^+$ T cells at 1 or 2 months prechallenge (left panel [$n = 17$; samples of macaque R01-008 prechallenge were unavailable]) and 1 week postchallenge (right panel [$n = 18$]). TNF-α^+ frequencies were significantly higher than those of any of the other four markers prechallenge, whereas $CD107a^+$ frequencies were significantly higher than those of TNF-α^+ and IL-2^+ at week 1 postchallenge (Friedman's test and Wilcoxon signed-rank test). (B) Comparison of SIV-specific $CD107a^+$, MIP-$1\beta^+$, IFN-γ^+, TNF-α^+, and IL-2^+ $CD4^+$ T-cell frequencies prechallenge (pre) and at week 1 (wk 1) in vaccinated animals ($n = 17$). Cutoff values are indicated by dotted lines (see Materials and Methods). No significant change in SIV-specific $CD107a^+$ $CD4^+$ T-cell frequencies was observed, whereas other subset frequencies were significantly reduced following challenge (MIP-$1\beta^+$, $P = 0.0005$; IFN-γ^+, $P = 0.0004$; TNF-α^+, $P = 0.0009$; IL-2^+, $P = 0.0005$ by Wilcoxon signed-rank test). (C) Comparison of SIV-specific $CD107a^-$ $CD4^+$ T-cell frequencies prechallenge and 1 week postchallenge in vaccinated macaques ($n = 17$). The prechallenge frequencies were significantly higher than those at week 1 ($P = 0.0005$ by Wilcoxon signed-rank test).

and a part of Vpr (see Materials and Methods). A representative gating schema for the flow cytometric analysis is shown in Fig. S2 in the supplemental material.

We first examined SIV-specific individual marker frequencies in total $CD4^+$ T cells 1 or 2 months before and 1 week after SIVmac239 challenge in vaccinated macaques (Fig. 1A). Multiple comparisons among the five markers prechallenge revealed that SIV-specific TNF-α^+ $CD4^+$ T-cell frequencies were the highest while $CD107a^+$ frequencies were the lowest. In contrast, SIV-specific $CD4^+$ T cells postchallenge showed a different hierarchy of individual marker frequencies, with the highest being $CD107a^+$ and IL-2^+ the lowest.

We then compared pre- and postchallenge SIV-specific CD107a, MIP-1β, IFN-γ, TNF-α, and IL-2 responses in $CD4^+$ T cells (Fig. 1B). Remarkably, frequencies of SIV-specific MIP-$1\beta^+$, IFN-γ^+, TNF-α^+, and IL-2^+ subsets were significantly reduced following challenge ($P = 0.0005$, $P = 0.0004$, $P = 0.0009$, and $P = 0.0005$, respectively), but no significant reduction was observed in SIV-specific $CD107a^+$ $CD4^+$ T-cell frequencies. SIV-specific TNF-α^+/IL-2^+ $CD4^+$ T-cell frequencies were above the cutoff values (see Materials and Methods) in all vaccinated animals at prechallenge but in only 4/17 at week 1 postchallenge. SIV-specific MIP-$1\beta^+$/IFN-γ^+ $CD4^+$ T-cell frequencies were above the cutoff in 13/17 and 14/17 animals, respectively, prechallenge but in only 5/17 postchallenge. SIV-specific $CD107a^-$ $CD4^+$ T-cell frequencies ($CD107a^-$ populations in SIV-specific MIP-$1\beta^+$, IFN-γ^+, TNF-α^+, or IL-2^+ $CD4^+$ T cells) were significantly reduced ($P = 0.0005$) (Fig. 1C). In contrast, SIV-specific $CD107a^+$ $CD4^+$ T-cell responses were above the cutoff in nine vaccinees prechallenge and in nine postchallenge. These results indicate that SIV-specific $CD4^+$ T cells producing MIP-1β, IFN-γ, TNF-α, and/or IL-2 are efficiently elicited by the DNA-prime/SeV-Gag-boost vaccination but are depleted in the acute phase postchallenge, whereas vaccine-elicited SIV-specific $CD4^+$ T cells expressing CD107a are resistant to depletion following SIV infection.

We further examined whether vaccine-elicited SIV-specific $CD4^+$ T cells producing MIP-$1\beta^+$, IFN-γ^+, TNF-α^+, or IL-2^+ together with CD107a are resistant to depletion postchallenge. SIV-specific $CD107a^+$ TNF-α^+ and $CD107a^+$ IL-2^+ $CD4^+$ T-cell frequencies were significantly reduced following SIV challenge ($P = 0.0125$ and $P = 0.0137$, respectively), whereas no significant reduction was observed in SIV-specific $CD107a^+$ MIP-$1\beta^+$ or $CD107a^+$ IFN-γ^+ $CD4^+$ T-cell subset (Fig. 2A). SIV-specific $CD107a^-$ MIP-$1\beta^+$, $CD107a^-$ IFN-γ^+, $CD107a^-$ TNF-α^+, and $CD107a^-$ IL-2^+ $CD4^+$ T-cell frequencies showed more profound and significant reductions following challenge ($P = 0.0005$, $P = 0.0001$, $P = 0.0011$, and $P = 0.0005$, respectively) (Fig. 2B). Comparison of $CD107a^+$ and $CD107a^-$ populations in SIV-specific TNF-α^+ and IL-2^+ $CD4^+$ T cells revealed that the latter ($CD107a^-$) subset was higher at prechallenge (Fig. 3A) whereas the former ($CD107a^+$) subset was predominant mostly in those that remained above the cutoff values at week 1 postchallenge (Fig. 3B). These results imply that vaccine-elicited $CD4^+$ T cells

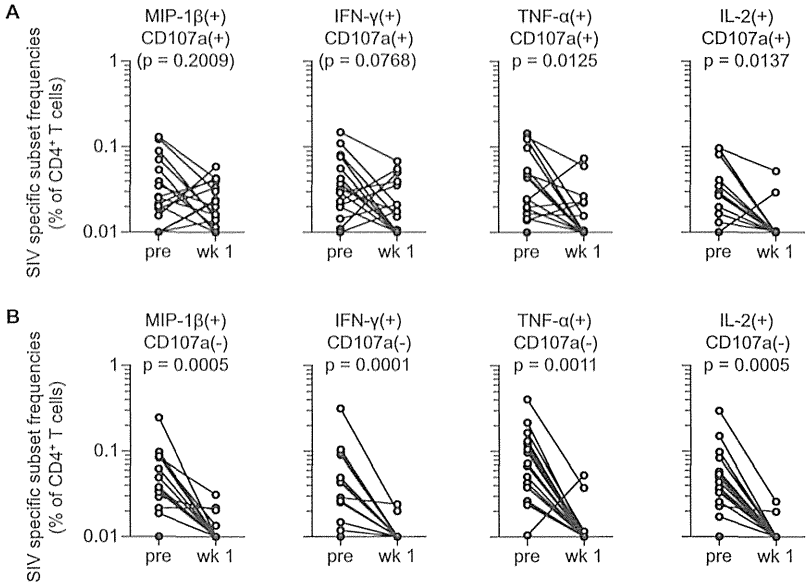

FIG 2 SIV-specific CD107a$^+$ and CD107a$^-$ CD4$^+$ T-cell responses pre- and postchallenge in vaccinated macaques. (A) Comparison of SIV-specific MIP-1β$^+$ CD107a$^+$, IFN-γ$^+$ CD107a$^+$, TNF-α$^+$ CD107a$^+$, and IL-2$^+$ CD107a$^+$ CD4$^+$ T-cell frequencies prechallenge and at week 1 postchallenge. No significant change in SIV-specific MIP-1β$^+$ or IFN-γ$^+$ CD107a$^+$ CD4$^+$ T-cell frequencies was observed, whereas frequencies of the other two subsets were significantly reduced following challenge (TNF-α$^+$, $P = 0.0125$; IL-2$^+$, $P = 0.0137$ by Wilcoxon signed-rank test). (B) Comparison of SIV-specific MIP-1β$^+$ CD107a$^-$, IFN-γ$^+$ CD107a$^-$, TNF-α$^+$ CD107a$^-$, and IL-2$^+$ CD107a$^-$ CD4$^+$ T-cell frequencies prechallenge and at week 1 postchallenge. All these frequencies were significantly reduced following challenge (MIP-1β$^+$, $P = 0.0005$; IFN-γ$^+$, $P = 0.0001$; TNF-α$^+$, $P = 0.0011$; IL-2$^+$, $P = 0.0005$ by Wilcoxon signed-rank test).

producing these markers together with CD107a are relatively resistant to depletion following SIV challenge.

SIV-specific CD4$^+$ T-cell responses pre- and postchallenge in vaccinated noncontrollers and controllers. Next, we compared SIV-specific CD4$^+$ T-cell responses in vaccinated noncontrollers (v-NC) and controllers (v-C). No significant difference was observed in SIV-specific CD107a$^+$, MIP-1β$^+$, IFN-γ$^+$, TNF-α$^+$, or IL-2$^+$ CD4$^+$ T-cell frequencies between these two groups before SIV challenge (Fig. 4A), indicating that prechallenge SIV-specific CD4$^+$ T-cell responses are not the major determinant for SIV control in these vaccinated animals.

In vaccinated noncontrollers, SIV-specific MIP-1β$^+$, IFN-γ$^+$, TNF-α$^+$, and IL-2$^+$ CD4$^+$ T cells were significantly reduced following SIV challenge, while reduction in SIV-specific CD107a$^+$

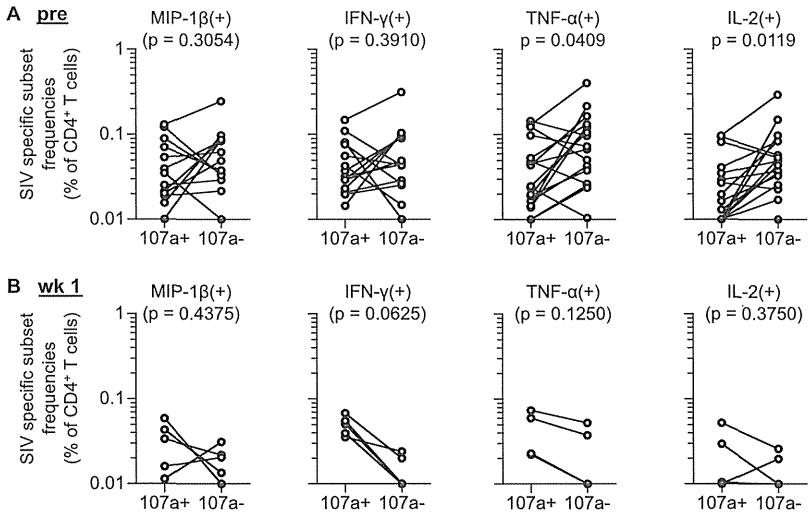

FIG 3 Comparison of SIV-specific CD107a$^+$ and CD107a$^-$ CD4$^+$ T-cell responses in vaccinated macaques. (A) Comparison of frequencies of prechallenge SIV-specific CD4$^+$ T-cell subsets inducing individual markers with (107a$^+$) and without CD107a (107a$^-$). Data for animals having SIV-specific MIP-1β$^+$ ($n = 13$), IFN-γ$^+$ ($n = 14$), TNF-α$^+$ ($n = 17$), and IL-2$^+$ ($n = 17$) CD4$^+$ T-cell frequencies above individual cutoff values are shown. (B) Comparison of frequencies of postchallenge SIV-specific CD4$^+$ T-cell subsets inducing individual markers with (107a$^+$) and without CD107a (107a$^-$). Data for animals having SIV-specific MIP-1β$^+$ ($n = 5$), IFN-γ$^+$ ($n = 5$), TNF-α$^+$ ($n = 4$), and IL-2$^+$ ($n = 4$) CD4$^+$ T-cell frequencies above individual cutoff values are shown.

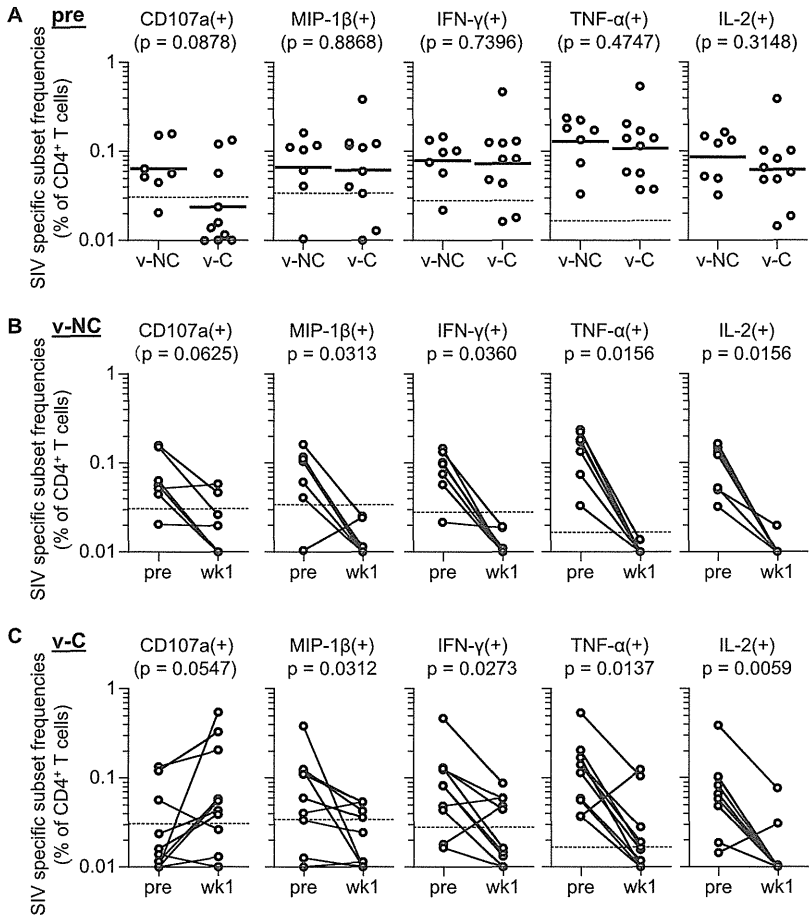

FIG 4 SIV-specific $CD4^+$ T-cell responses pre- and postchallenge in vaccinated noncontrollers (v-NC) and controllers (v-C). (A) Comparison of SIV-specific $CD107a^+$, MIP-$1\beta^+$, IFN-γ^+, TNF-α^+, and IL-2^+ $CD4^+$ T-cell frequencies prechallenge in v-NC ($n = 7$) and v-C ($n = 10$; samples of macaque R01-008 prechallenge were unavailable]). No significant difference was detected between the two groups for any of the 5 markers. (B) Comparison of SIV-specific $CD107a^+$, MIP-$1\beta^+$, IFN-γ^+, TNF-α^+, and IL-2^+ $CD4^+$ T-cell frequencies prechallenge and at week 1 postchallenge in v-NC. SIV-specific MIP-$1\beta^+$, IFN-γ^+, TNF-α^+, and IL-2^+ $CD4^+$ T-cell frequencies were significantly reduced following challenge (MIP-$1\beta^+$, $P = 0.0313$; IFN-γ^+, $P = 0.0360$; TNF-α^+, $P = 0.0156$; IL-2^+, $P = 0.0156$ by Wilcoxon signed-rank test). (C) Comparison of SIV-specific $CD107a^+$, MIP-$1\beta^+$, IFN-γ^+, TNF-α^+, and IL-2^+ $CD4^+$ T-cell frequencies prechallenge and at week 1 postchallenge in v-C. SIV-specific MIP-$1\beta^+$, IFN-γ^+, TNF-α^+, and IL-2^+ $CD4^+$ T cells were significantly reduced following challenge (MIP-$1\beta^+$, $P = 0.0312$; IFN-γ^+, $P = 0.0273$; TNF-α^+, $P = 0.0137$; IL-2^+, $P = 0.0059$ by Wilcoxon signed-rank test).

$CD4^+$ T cells was not significant (Fig. 4B). SIV-specific MIP-$1\beta^+$, IFN-γ^+, TNF-α^+, and IL-2^+ $CD4^+$ T-cell frequencies at 1 week postchallenge were below the cutoff values in almost all noncontrollers, and even the $CD107a^+$ subsets were below the cutoff in five of the seven. In contrast, SIV-specific $CD107a^+$ $CD4^+$ T-cell frequencies were not reduced but rather increased following challenge in vaccinated controllers; 7 of the 10 showed an increase in SIV-specific $CD107a^+$ $CD4^+$ T-cell responses (Fig. 4C). MIP-$1\beta^+$, IFN-γ^+, TNF-α^+, and IL-2^+ subsets postchallenge were above the cutoff in 5/10, 5/10, 4/10, and 3/10, respectively, although significant reductions in these subset frequencies were observed. Thus, reductions in vaccine-elicited SIV-specific $CD4^+$ T cells following SIV challenge were prominent in noncontrollers but not in controllers.

Comparison of SIV-specific $CD4^+$ T-cell responses postchallenge in unvaccinated animals, vaccinated noncontrollers, and vaccinated controllers. We then examined SIV-specific individual marker responses in $CD4^+$ T cells at week 1 postinfection in unvaccinated macaques (Fig. 5A). Unvaccinated animals showed a higher frequency of SIV-specific $CD107a^+$ $CD4^+$ T cells than other markers, as seen in vaccinees at week 1 postchallenge (Fig. 1A), implying that the $CD107a^+$ subset in unvaccinated animals may also be relatively resistant to depletion in the acute phase of SIV infection.

Next, we compared SIV-specific $CD4^+$ T-cell responses at 1 week postchallenge in unvaccinated animals, vaccinated noncontrollers, and vaccinated controllers (Fig. 5B). No significant difference in SIV-specific $CD107a^+$ $CD4^+$ T-cell responses was observed among these groups, but there was a trend for a lower frequency of this subset in vaccinated noncontrollers. SIV-specific $CD107a^+$ $CD4^+$ T-cell frequencies were above the cutoff values in 10 of 21 unvaccinated animals and 7 of 11 vaccinated controllers but only in 2 of 7 vaccinated noncontrollers. SIV-specific MIP-$1\beta^+$, IFN-γ^+, TNF-α^+, and IL-2^+ $CD4^+$ T cells were below the cutoff in almost all vaccinated noncontrollers. Thus, SIV-specific $CD4^+$ T-cell depletion occurred primarily following SIV challenge in vaccinated noncontrollers.

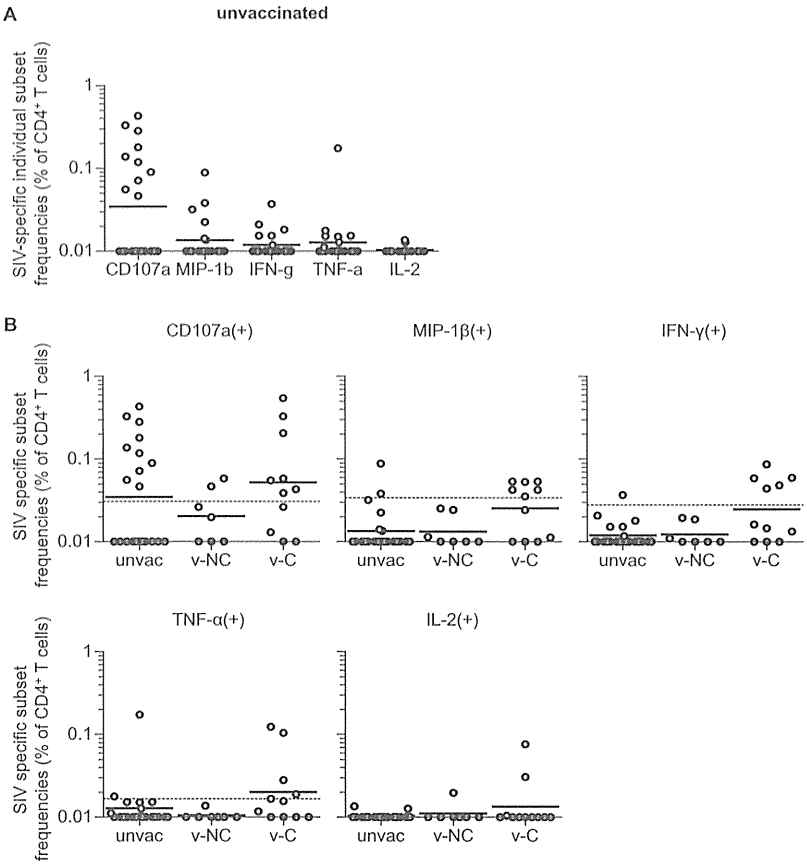

FIG 5 SIV-specific CD4$^+$ T-cell responses at week 1 postchallenge in unvaccinated and vaccinated macaques. (A) SIV-specific CD107a$^+$, MIP-1β$^+$, IFN-γ$^+$, TNF-α$^+$, and IL-2$^+$ frequencies in CD4$^+$ T cells in unvaccinated macaques (n = 21). No significant difference was indicated by multiple comparisons (Friedman's test and Wilcoxon signed-rank test). (B) SIV-specific CD107a$^+$, MIP-1β$^+$, IFN-γ$^+$, TNF-α$^+$, and IL-2$^+$ CD4$^+$ T-cell frequencies in unvaccinated animals (unvac; n = 21), vaccinated noncontrollers (v-NC; n = 7), and vaccinated controllers (v-C; n = 11).

Comparison of plasma viral loads in the acute phase in unvaccinated, vaccinated noncontroller, and controller groups. Finally, we compared plasma viral loads in the acute phase in unvaccinated, vaccinated noncontroller, and controller groups. Interestingly, vaccinated noncontrollers showed significantly higher viral loads at week 1 than unvaccinated as well as vaccinated controllers (Fig. 6A). Even compared to the unvaccinated noncontrollers, vaccinated noncontrollers had significantly higher viral loads at week 1 (Fig. 6B). Unvaccinated but not vaccinated animals showed a significant increase in viral loads from week 1 to week 2 postchallenge (Fig. 6C), indicating that viral loads peaked earlier in vaccinated macaques. At week 2, unvaccinated animals had viral loads that were at levels similar to those of vaccinated noncontrollers but significantly higher than those of vaccinated controllers (Fig. 6A). These results suggest a higher acceleration of viral replication in the acute phase following SIV infection in vaccinated noncontrollers than in unvaccinated animals.

DISCUSSION

Virus-specific CD4$^+$ T-cell responses are crucial for induction of effective antibody and CD8$^+$ T-cell responses against virus infection. Current vaccine strategies include induction of neutralizing antibody and/or CD8$^+$ T-cell responses, which are accompanied by CD4$^+$ T-cell induction. Vaccine-induced CD4$^+$ T cells, however, can be the preferential targets for HIV/SIV infection. In the present study, we found that vaccine-elicited SIV-specific CD107a$^-$ CD4$^+$ T cells are depleted in the acute phase of infection after SIV challenge. In contrast, our results indicate that SIV-specific CD4$^+$ T cells expressing CD107a are relatively resistant to depletion following infection.

HIV is known to preferentially infect HIV-specific CD4$^+$ T cells (32). Our results present the basis of this preference. However, the mechanism of relative resistance of the CD107a$^+$ population in HIV/SIV-specific CD4$^+$ T cells to depletion following infection remains undetermined. Analysis using PBMCs found no significant difference in CCR5$^+$ frequencies among SIV-specific CD107a$^+$, MIP-1β$^+$, IFN-γ$^+$, TNF-α$^+$, and IL-2$^+$ CD4$^+$ T cells (see Fig. S3 in the supplemental material). CD107a$^+$ subset frequencies were the lowest among the five markers after vaccination (Fig. 1A), and if this subset's responses were also lower following infection, it may contribute to lower sensitivity to depletion. It is difficult, however, to examine *in vitro* SIV infection and T-cell responses under the conditions exactly reflecting what occurs *in vivo*. It is also difficult to determine the possibility of changes in SIV-specific CD4$^+$ T-cell function following infection.

It has been reported that virus-specific CD107a expression in

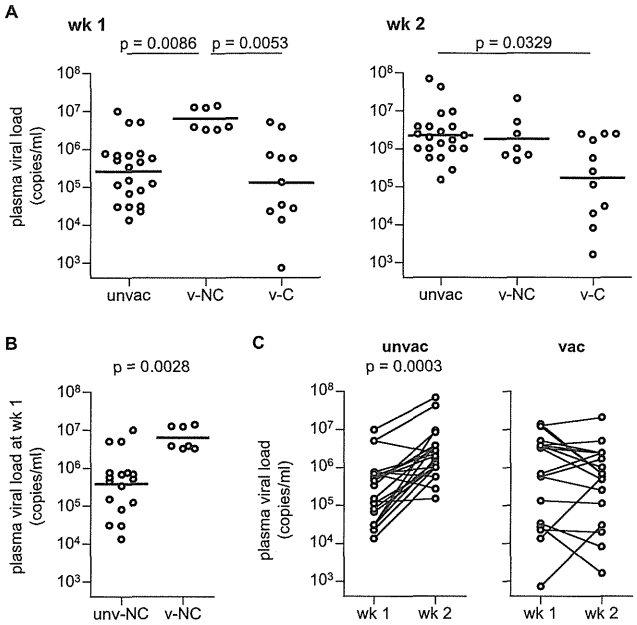

FIG 6 Plasma viral loads at weeks 1 and 2 after SIVmac239 challenge. (A) Comparison of viral loads in unvaccinated animals (unvac), vaccinated noncontrollers (v-NC), and vaccinated controllers (v-C) at weeks 1 (left panel) and 2 (right panel). Multiple comparisons (Kruskal-Wallis test and Mann-Whitney U test) indicated significantly higher viral loads at week 1 in v-NC than unvac and v-C ($P = 0.0086$ and $P = 0.0053$, respectively) and significantly lower viral loads at week 2 in v-C than unvac ($P = 0.0329$). (B) Comparison of viral loads at week 1 between unvaccinated noncontrollers (unv-NC; $n = 17$) and vaccinated noncontrollers (v-NC; $n = 7$). The load for the latter set was significantly higher than for the former ($P = 0.0028$ by Mann-Whitney U test). (C) Comparison of viral loads between weeks 1 and 2 in unvaccinated (left panel) and vaccinated (right panel) animals. Unvaccinated animals showed significantly higher viral loads at week 2 than week 1 ($P = 0.0003$ by Wilcoxon signed-rank test).

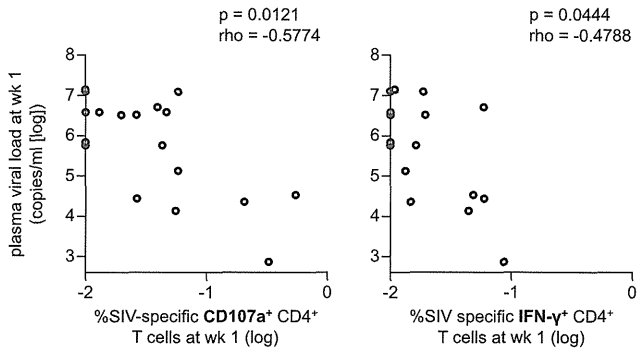

FIG 7 Correlation analysis between SIV-specific $CD4^+$ T-cell frequencies at week 1 and plasma viral loads at week 1. SIV-specific $CD107a^+$ (left panel) and IFN-γ^+ (right panel) $CD4^+$ T-cell frequencies were inversely correlated with the viral loads ($P = 0.0121$, $rho = -0.5774$ for the left panel and $P = 0.0444$, $rho = -0.4788$ for the right panel by Spearman's test).

$CD4^+$ T cells is associated with cytotoxic $CD4^+$ T-cell function via cytotoxic granules (46–49), which may confer resistance. Virus-specific MIP-1β^+, IFN-γ^+, TNF-α^+, and IL-2^+ rather than $CD107a^+$ $CD4^+$ T cells are believed to be important for helper function (27, 36). In particular, IFN-γ is an important marker for T_H1 cells. However, our results indicate that vaccine-induced $CD4^+$ T cells producing MIP-1β, IFN-γ, TNF-α, or IL-2 are largely depleted following SIV challenge. SIV-specific TNF-α^+ and IL-2^+ populations decreased postchallenge even in $CD107a^+$ $CD4^+$ T cells, suggesting that these TNF-α and IL-2 responses may confer higher sensitivity to depletion on $CD4^+$ T cells. Nevertheless, the reduction of the $CD107a^+$ population postchallenge was less prominent than that of $CD107a^-$ in SIV-specific TNF-α^+ and IL-2^+ $CD4^+$ T cells. Furthermore, the $CD107a^+$ population of SIV-specific MIP-1β^+ or IFN-γ^+ $CD4^+$ T cells showed no significant reduction postchallenge. These results imply that the $CD107a^+$ subset of vaccine-elicited $CD4^+$ T cells with helper function may be relatively resistant to depletion following HIV/SIV infection.

Our previous studies (33, 34, 39) showed that vaccine-induced Gag-specific $CD8^+$ T-cell responses are responsible for the control of SIV replication in the vaccinated controllers used in the present study. No significant difference in prechallenge SIV-specific $CD4^+$ T-cell responses was observed between vaccinated controllers and noncontrollers, supporting a notion that vaccine-induced $CD4^+$ T-cell responses are not the determinant for SIV control in these animals. There was no correlation between prechallenge SIV-specific $CD107a^+$, MIP-1β^+, IFN-γ^+, TNF-α^+, or IL-2^+ $CD4^+$ T-cell frequencies and viral loads at week 1. However, the noncontrollers showed a larger reduction in SIV-specific $CD4^+$ T cells following SIV challenge and higher plasma viral loads at week 1 than the controllers. Even the $CD107a^+$ as well as IFN-γ^+ subset frequencies at week 1 were inversely correlated with viral loads at week 1 postchallenge in vaccinated animals (Fig. 7). These results imply that the reduction of vaccine-induced SIV-specific $CD4^+$ T cells reflects killing of these cells by SIV within 1 week postchallenge. Vaccine-induced $CD4^+$ T cells would be subjected to the killing without effectors such as $CD8^+$ T cells, which protect these cells following infection.

SIV-specific MIP-1β^+, IFN-γ^+, TNF-α^+, and IL-2^+ $CD4^+$ T cells were mostly depleted at week 1 in vaccinated noncontrollers. We found that viral loads peaked earlier in vaccinated than in unvaccinated animals. Furthermore, vaccinated noncontrollers that showed depletion of vaccine-elicited $CD4^+$ T cells had significantly higher viral loads at week 1 than unvaccinated animals. While virus-specific $CD4^+$ T-cell responses are important in viral control (50–52), our results suggest that induction of virus-specific $CD4^+$ T cells, especially $CD107a^-$ cells, by vaccination may not lead to efficient $CD4^+$ T-cell responses following infection but rather enhance or accelerate viral replication in the early acute phase after HIV/SIV exposure. It is speculated that vaccinated controllers elicited highly effective $CD8^+$ T-cell responses, which could overwhelm this enhanced viral replication. Without this enhancement, however, such highly potent effectors may not be required for HIV/SIV control. Thus, it would be reasonable to develop a vaccine to induce effective responses without inducing HIV-specific memory $CD107a^-$ $CD4^+$ T cells. Indeed, our previous study suggested that vaccine induction of epitope-specific $CD8^+$ T cells with the help of SeV-specific but not SIV-specific $CD4^+$ T cells can result in effective $CD8^+$ T-cell responses against SIV infection in the acute phase postchallenge (53). Alternatively, induction of HIV-specific $CD107a^+$ $CD4^+$ T cells may be a promising HIV vaccine approach, although the strategy for induction of these cells remains unknown (27, 54).

In summary, this study found that vaccine-elicited SIV-specific

CD4$^+$ T cells expressing CD107a are relatively resistant to depletion following infection in a macaque AIDS model. In contrast, our analysis revealed massive depletion of SIV-specific CD107a$^-$ CD4$^+$ T cells following SIV exposure. These results suggest a rationale for vaccine design to elicit effective antibody or CD8$^+$ T-cell responses without induction of HIV-specific CD107a$^-$ CD4$^+$ T cells toward HIV control.

ACKNOWLEDGMENTS

This work was supported by a grant-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology in Japan and grants-in-aid from the Ministry of Health, Labor, and Welfare in Japan.

We thank F. Ono, K. Oto, K. Hanari, S. Okabayashi, H. Akari, Y. Yasutomi, T. Miura, and Y. Koyanagi for their assistance in animal experiments. We also thank M. de Souza for his comments on the manuscript.

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Switching and emergence of CTL epitopes in HIV-1 infection

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Abstract

Background: Human Leukocyte Antigen (HLA) class I restricted Cytotoxic T Lymphocytes (CTLs) exert substantial evolutionary pressure on HIV-1, as evidenced by the reproducible selection of HLA-restricted immune escape mutations in the viral genome. An escape mutation from tyrosine to phenylalanine at the 135th amino acid (Y135F) of the HIV-1 *nef* gene is frequently observed in patients with HLA-A*24:02, an HLA Class I allele expressed in ~70% of Japanese persons. The selection of CTL escape mutations could theoretically result in the *de novo* creation of novel epitopes, however, the extent to which such dynamic "CTL epitope switching" occurs in HIV-1 remains incompletely known.

Results: Two overlapping epitopes in HIV-1 *nef*, Nef126-10 and Nef134-10, elicit the most frequent CTL responses restricted by HLA-A*24:02. Thirty-five of 46 (76%) HLA-A*24:02-positive patients harbored the Y135F mutation in their plasma HIV-1 RNA. Nef codon 135 plays a crucial role in both epitopes, as it represents the C-terminal anchor for Nef126-10 and the N-terminal anchor for Nef134-10. While the majority of patients with 135F exhibited CTL responses to Nef126-10, none harboring the "wild-type" (global HIV-1 subtype B consensus) Y135 did so, suggesting that Nef126-10 is not efficiently presented in persons harboring Y135. Consistent with this, peptide binding and limiting dilution experiments confirmed F, but not Y, as a suitable C-terminal anchor for HLA-A*24:02. Moreover, experiments utilizing antigen specific CTL clones to recognize endogenously-expressed peptides with or without Y135F indicated that this mutation disrupted the antigen expression of Nef134-10. Critically, the selection of Y135F also launched the expression of Nef126-10, indicating that the latter epitope is created as a result of escape within the former.

Conclusions: Our data represent the first example of the *de novo* creation of a novel overlapping CTL epitope as a direct result of HLA-driven immune escape in a neighboring epitope. The robust targeting of Nef126-10 following transmission (or *in vivo* selection) of HIV-1 containing Y135F may explain in part the previously reported stable plasma viral loads over time in the Japanese population, despite the high prevalence of both HLA-A*24:02 and Nef-Y135F in circulating HIV-1 sequences.

Background

Cytotoxic T lymphocytes (CTLs) are key players in the immune control of Human Immunodeficiency Virus 1 (HIV-1), as they recognize virally-derived peptide epitopes presented by HLA class I molecules on the infected cell surface [1,2]. Over the course of infection however, HIV-1 mutations arise within the infected individual, notably in targeted CTL epitopes, that allow the virus to escape immune recognition by CTLs. Importantly, despite the hypermutability of HIV-1, these immune escape mutations often arise in a stereotypical manner [3,4] that is highly predictable based on the specific HLA class I molecules expressed by the host [5-8]. Although selection of HLA-associated mutations in HIV-1 is driven by immune pressure, these amino acid substitutions sometimes result in the induction of a *de novo* immune response in which the mutant epitope is recognized by a TCR associated with a different CTL subset [7,9]. What is less well-characterized is the

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extent to which selection of immune escape mutations result in the *de novo* creation of novel CTL epitopes nearby, that could subsequently be targeted by CTL *in vivo* (in a manner similar to the continual exposure of novel antibody epitopes in HIV-1 envelope as a consequence of escape from earlier humoral responses [10]). Here, we demonstrate such a dynamic phenomenon of "CTL epitope switching" as a direct result of CTL escape from HLA-A*24:02.

We reported previously that the substitution from tyrosine to phenylalanine (Y135F) at the 135th amino acid of the HIV-1 *nef* gene is frequently observed in patients with HLA-A*24:02, an HLA Class I allele expressed in ~70% of Japanese persons [4,11]. Our observation that Y135F appeared to be an escape mutation was later confirmed [12]. In order to examine the influence of HIV-1 mutations on the strength of various epitope-specific CTL responses, we studied CTL epitopes restricted by HLA-A*24:02 in relatively conserved regions of the HIV-1 genome. Our results indicate that Nef-Y135F, selected to escape recognition of a well-described HLA-A*24:02-restricted CTL epitope in this viral protein, results in the creation of another HLA-A*24:02 epitope immediately upstream. To our knowledge, our findings represent the first evidence of immune escape-driven "epitope switching" in HIV-1 infection.

Results

Identification of immunodominant CTL responses restricted by HLA-A*24:02

Forty-six HLA-A*24:02-positive patients with HIV-1 infection were studied. Forty-four were infected through unprotected sexual intercourse and 2 were hemophiliacs. Forty-five were infected with subtype B except one was infected with subtype AG. The median plasma viral load (pVL) was 4.11 (range 2.26 to 5.36) log 10 copies/ml, and the median CD4 cell count was 395 (range 120 to 1,035) cells/μl. To determine which published HLA-A*24:02-restricted CTL epitopes are most frequently recognized among persons expressing this allele, IFN-γ ELISpot assays were performed using expanded PBMCs. Due to limited PBMC numbers, 11 published A*24:02-restricted CTL epitopes in the relatively conserved *gag*, *pol* and *nef* regions [13-15] were selected for investigation. Published optimal epitopes were used for the assay. The response rate against Nef134-10 was highest (80.4%), followed by Nef126-10 (50.0%), Gag28-9 (40.0%) and Pol496-9 (28.3%), while limited (<10%) or no responses were observed in the other epitopes (Figure 1A, B). Of note, Nef126-10 and Nef134-10 overlap each other by 2 amino acids (Figure 1A).

We next analyzed patient plasma HIV RNA amino acid sequences within the Nef126-10–Nef134-10 regions (Table 1). The great majority of patients (35/46 = 76.1%) had a tyrosine (Y) to phenylalanine (F) mutation (Y135F) at Nef codon 135 (Nef135F) while eight patients (8/46 = 17.4%) had the global consensus subtype B residue at this position (Nef135Y). Two were Nef135L and one was Nef135W. These results were consistent with our earlier findings [4]. Intriguingly, none of the eight patients with Nef135Y exhibited a Nef126-10-specific response, while all of them exhibited a Nef134-10-specific response ($p < 0.001$, Fisher's exact test) (Figure 1C, left). Of the 35 patients harboring Nef135F, 23 (65.7%) and 28 (80.0%) responded to Nef126-10 and Nef134-10, respectively (Figure 1C, right) ($p = 0.2823$, Fisher's exact test).

Dramatic improvement in the HLA-binding affinity of Nef126-10 following mutation of the C-terminal anchor residue

To clarify the relationship between Y135F and peptide-specific responses, we examined HLA-binding affinity of the wild type and mutant peptides using *in vitro* peptide-HLA binding assays (Figure 2A). In context of the Nef134-10 epitope, the mutant Y135F peptide (representing position 2, the N-terminal anchor of this epitope; Nef134-10(2F)) was almost as effective as the "wild type" Nef134-10 (Nef134-10(wt)) peptide in binding to HLA-A*24:02. In contrast, in context of the Nef126-10 epitope, the mutant Y135F peptide (representing position 10, the C-terminal anchor of this epitope), dramatically improved its binding to HLA-A*24:02. The presence of threonine (T) at the 8th position (Nef126-10(8T10F)), representing Nef mutation I133T, did not significantly affect epitope-HLA binding compared to the wild type isoleucine (I) (Nef126-10(8I10F)). These results are compatible with previous reports identifying Y or F as possible N-terminal anchors for HLA-A*24:02, but only F as a possible C-terminal anchor [16,17].

We then examined the effect of the mutations on epitope recognition using CTL clones established from patients with HIV-1 infection. 293FT-A24DRm-CY0 cells pulsed with different dilutions of peptides were co-cultured with CTL cell clones. The Nef134-10-specific CTL clone H27-9 produced IFN-γ almost equally well in response to Nef134-10(wt) peptides or to Nef134-10 (2F) peptides (Figure 2B, left). In contrast, the Nef126-10-specific CTL clone I30-1 produced IFN-γ only at high concentrations of the wild-type Nef126-10(wt) peptide, whereas mutant peptides Nef126-10(8I10F) and Nef126-10(8T10F) induced strong responses at very low peptide concentration (Figure 2B, right). These results were consistent with peptide-HLA binding assays suggesting that the I133T mutation did not have much effect on recognition of the epitope-HLA complex by the Nef126-10-specific CTL clone I30-1. Moreover, the results were consistent with the observation that the presence of

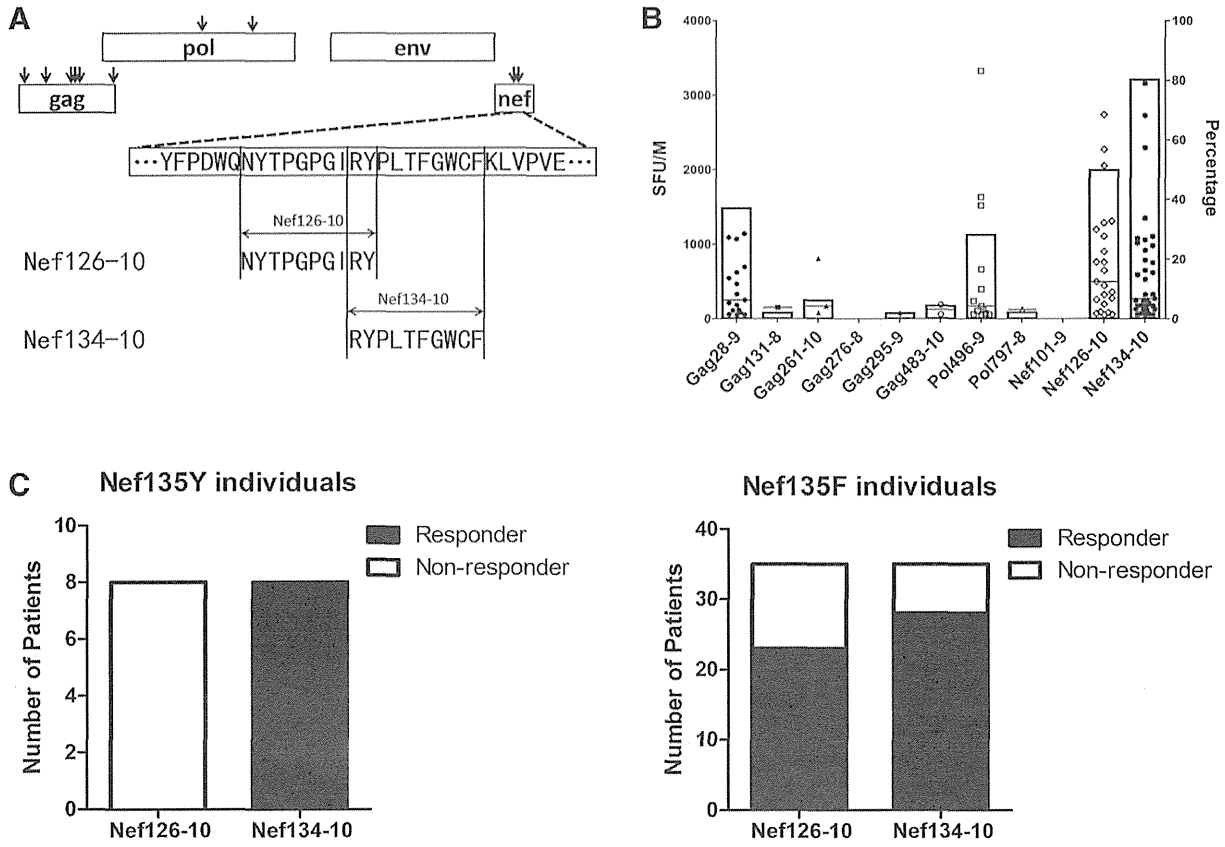

Figure 1 The association between the two overlapping epitopes and the Y135F mutation. (A) HIV-1 map of representative proteins and the locations of HLA-A*24:02-restricted epitopes used in this study are shown (arrow). The positional relation and the sequence information around Nef126-10 and Nef134-10 epitopes are also shown. **(B)** Immune responses to 11 epitopes were assessed by IFN-γ ELISpot assay by using expanded PBMCs from 46 HIV-1 patients. Epitope names below the chart indicate peptides used in the assay; [Protein][Location(HXB2 numbering)]-[Amino acid length]. Each point represents the average SFU of duplicate wells after subtraction of background in each individual. Each stick represents response rate in 46 individuals. **(C)** The Nef126-10/Nef134-10-specific response rates among individuals harboring either Nef135Y or Nef135F were assessed. Black bars indicate number of responders to each epitope, and white bars for non-responders.

wild-type Y at the C-terminus lowers the affinity of the Nef126-10 peptide to HLA-A*24:02 (Figure 2A).

CTL responses against the endogenously expressed epitopes

In order to examine whether intracellularly-derived Nef protein could still be targeted by peptide-specific CTLs, we constructed *nef*-minigene expression vectors, pmNef (wt)-hRluc-EGFP, pmNef(135F)-hRluc-EGFP, and pmNef (133T135F)-hRluc-EGFP, for the generation of polypeptides encompassing the Nef126-10 and Nef134-10 epitopes (Figure 3A). The vectors encoded EGFP as a transfection marker, as well as the *Renilla* Luciferase (Rluc) gene hooked to the mini-*nef* gene by a GlyGlyGly-GlySer linker. Rluc activity served as a quantitative reference for the expression of the mini-*nef* polypeptide. Each vector was transfected into 293FT-A24DRm-CY0 cells. Rluc activities indicated that three types of *nef*-minigenes were expressed well and to comparable levels (Figure 3B).

We and others reported previously that Y135F is a processing mutation, as CTL responses could be induced to mutant epitopes via peptide-pulsing, but not via intracellularly-expressed polypeptide [4,12,18]. Consistent with the previous results, Nef134-10-specific responses by CTL clone H27-9 were induced by the wild type minigene, but diminished to minimal levels by the presence of Y135F or I133T/Y135F (Figure 3C, left).

By contrast, Nef126-10-specific responses by CTL clone I30-1 were provoked dramatically by the presence of Nef135F. Specifically, the Nef126-10-specific CTL clone I30-1 showed much higher responses to antigen-presenting cells transfected with the 133I/135F or 133T/135F minigene than Nef134-10-specific CTL clone H27-9. The I30-1 responses to minigenes encoding I versus T at the Nef133 position did not substantially differ (Figure 3C, right). In contrast, I30-1 responses to the wild type

Table 1 Amino acid sequences (Nef126-143) of plasma HIV-1 in 46 patients

Group[a]	Amino acid sequence[b]	Frequency (number)[c]
Consensus B	NYTPGPGIRYPLTFGWCF	
135Y group		*17.4% (8)*
	.....Y	2.2% (1)
	.....L....	4.3% (2)
	.....L...Y	2.2% (1)
	.....V.....	2.2% (1)
	C.....T.....L....	2.2% (1)
	.....T.....P.	2.2% (1)
	.....T....C.....	2.2% (1)
135F group		*76.1% (35)*
	.....F.....	4.3% (2)
	C.....F.....	2.2% (1)
	G.....F.....	2.2% (1)
	.....F..C.....	2.2% (1)
	.....T.F.....	50.0% (23)
	C.....T.F.....	2.2% (1)
	G.....T.F.....	2.2% (1)
	.....V.F.....	8.7% (4)
	.....E.F..C.....	2.2% (1)
others		*6.5% (3)*
	.....V.L.....	4.3% (2)
	.....T.W.....	2.2% (1)

[a]Patients were partitioned into three groups, Nef135Y (135Y), Nef135F (135F), or others, according to their amino acid information at the Nef135 position.
[b]Middle column shows amino acid sequence of the Nef126-143 region. The same amino acids as the subtype B consensus sequence are indicated by dots. Differences compared to the subtype B consensus sequence are indicated by the corresponding letters.
[c]Right column indicates frequency (and number) of individuals exhibiting the stated sequence. Subtotal frequency (and number) of each group is italicized.

minigene were indistinguishable from background. These results suggest that wild-type Nef126-10 peptide was not expressed as an epitope on the surface of the antigen-presenting cells when expressed endogenously, but Nef126-10 containing 135F (regardless of variation at position 133) was efficiently expressed. In turn, these *in vitro* results (Figure 2 and 3) strongly suggest that a novel mechanism, i.e. "epitope switching" was taking place after the selection of the Y135F mutation *in vivo* (Figure 1). Namely, selection of Y135F facilitates escape from CTL responses targeting the first epitope (Nef134-10), but simultaneously results in the creation of another epitope upstream (Nef126-10).

"Epitope switching" during the clinical course of HIV-1 infection

Among 8/46 patients in the IMSUT cohort who initially harbored the "wild type" (global consensus B) Y135 residue within the Nef134-10 epitope, we identified one patient who subsequently selected 135F, followed by 133T, over a period of 12 months. We performed IFN-γ ELISpot assays on PBMCs expanded from corresponding frozen longitudinal samples (Figure 4A). Before the mutated viruses became the majority, specific responses to Nef134-10(wt) were the most prominent, followed by responses to Nef134-10(2F) (Figure 4B, left). Importantly, no Nef126-10-specific responses were observed at these time points. After plasma viruses were replaced by viruses with 133T/135F, robust responses against Nef126-10(8I10F) and Nef126-10(8T10F) were observed, while responses against Nef126-10(wt) were detected only at high peptide concentrations (Figure 4B, right). These results were consistent with the results *in vitro* using CTL clones (Figure 2B, right), and support the *in vivo* presentation of Nef126-10 only after selection of Y135F. Of interest, responses against Nef134-10 peptides decreased but remained detectable after the selection of 135F and 133T/135F mutations.

Coupled selection of Nef135F and Nef133T mutants in vivo

We investigated the correlation between Nef135F and Nef133T *in silico* in two other independent cohorts. In a large cohort of antiretroviral-naïve patients chronically infected with subtype B HIV-1 in British Columbia, Canada (British Columbia HOMER cohort), positive correlations between Nef135F and Nef133T (Odds ratio: 11.3), as well as between Nef135Y and Nef133I (Odds ratio: 16.3) were observed (Figure 5A, all $p < 0.0001$). Furthermore, in a multicenter longitudinal acute/early infection cohort comprising 16 HLA-A*24:02-expressing persons infected with subtype B HIV-1, selection of Nef135F preceded that of Nef133T by a short duration (Figure 5B). The median times to Y135F and I133T selection were 220 and 236 days, respectively, a difference that was not statistically significant.

The correlation between the magnitude of Nef126-10 (8I10F) or Nef126-10(8T10F)-specific response and pVL was assessed in 24 IMSUT cohort participants for whom Nef126-10(8I10F) and Nef126-10(8T10F) responses (measured by IFN-γ ELISpot) and pVL at the corresponding time point, were available (Figure 5C). Interestingly, Nef126-10(8I10F)-specific but not Nef126-10(8T10F)-specific responses were inversely correlated with pVL, suggesting that responses to the former, but not the latter, contribute to *in vivo* immune control.

Crystal structures of Nef126-10 epitopes presented on HLA-A*24:02

In order to examine the impact of these mutations on epitope structure, we solved the crystal structures of HLA-A24/Nef126-10(8I10F) and HLA-A24/Nef126-10

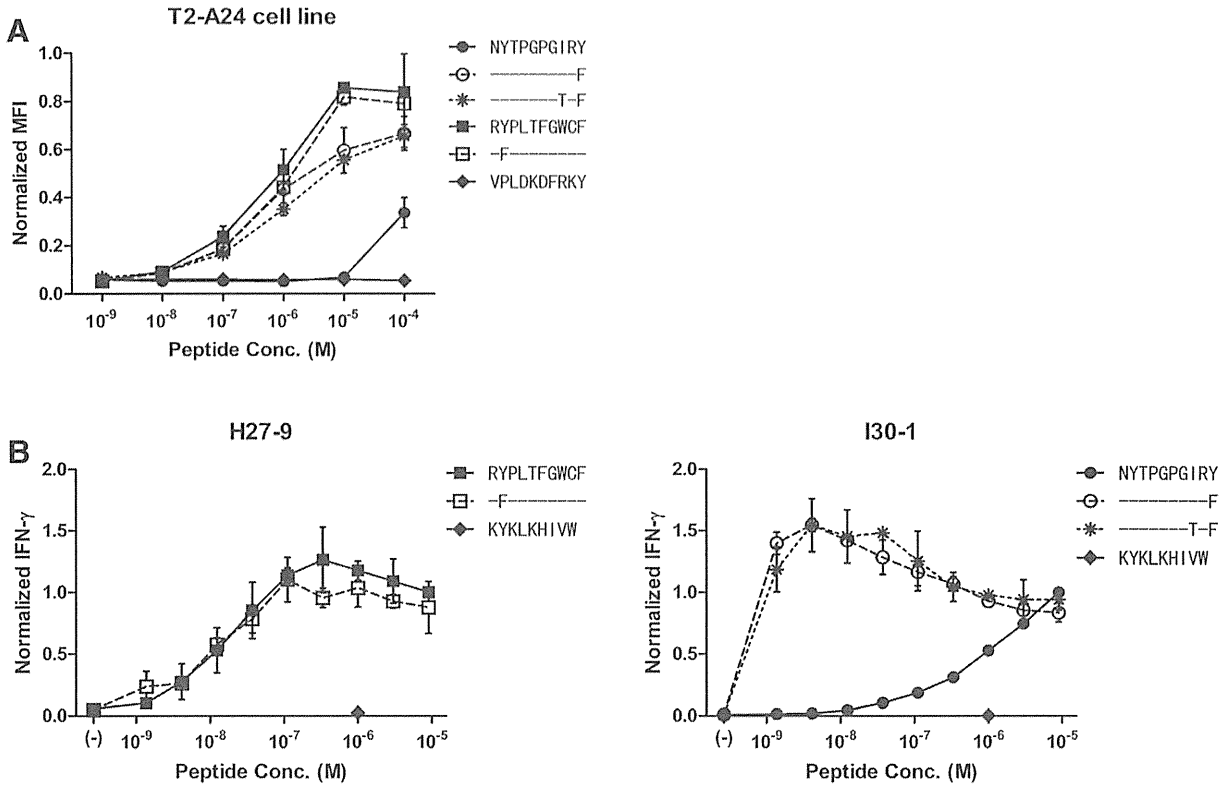

Figure 2 Epitope characterization. (A) Binding affinity of each peptide to HLA-A*24:02 was tested by using T2-A24 cell line. All peptides except negative control peptide, VPLDKDFRKY, which might not bind to HLA-A*24:02, show HLA-A*24:02 binding in the peptide concentration of 10^{-4} M. Each point and bar represents the average and the standard deviation of normalized MFI from three independent experiments. **(B)** 293FT-A24DRm-CY0 cells were pulsed with serially diluted peptides and co-cultured with CTL clones specific to Nef134-10 (H27-9) or Nef126-10 (I30-1). Epitope recognition was determined by normalizing quantity of IFN-γ secretion. For normalization, all values were divided by IFN-γ secretion with wild type peptide concentration of 9 μM. Neither clone showed specificity to the negative control, Gag28-9 peptide (KYKLKHIVW) a binder of HLA-A*24:02. Each point and bar show average and standard deviation of three independent experiments.

(8T10F) at 1.66 Å and 2.0 Å resolution, respectively (Figure 6A top and bottom; Additional file 1: Figure S1A, B). Superposition of the Nef126-10(8I10F) and Nef126-10(8T10F) peptide structures showed almost similar backbone atoms, with root mean square deviation of 0.307 Å, but conformational differences were found at P6 (131P) and P9 (134R) residues. The side chains of P6 and P9 residues in the Nef126-10(8I10F) and Nef126-10(8T10F) epitopes had poor electron densities in spite of structures being at modestly higher resolution (Additional file 1: Figure S1C, D). In addition, the B-factors for the central portions (P5-P7) of each peptide (41.5 $Å^2$ for the Nef126-10(8I10F) and 46.2 $Å^2$ for Nef126-10(8T10F)) were higher than for overall peptides (24.1 $Å^2$ for Nef126-10(8I10F) and 33.2 $Å^2$ for Nef126-10(8T10F)). These results indicated a flexibility of the central portion and P9 residue in both peptides, accounting for the structural difference observed.

The side chains of P8-Ile and P8-Thr protruded from, rather than being buried within, the antigen-binding cleft of HLA-A*24:02, suggesting the P8 residue could be involved in the contact with TCR (Figure 6A bottom). Therefore, different TCRs could be favored by the presence of either hydrophobic P8-I or hydrophilic P8-T at the interface of a TCR-HLA-A*24:02/Nef126-10. If this is the case, different TCR repertoires would be selected by Nef126-10(8I10F) or Nef126-10(8T10F), suggesting Nef-I133T as a possible immune escape mutation that alters the *in vivo* repertoire of CTL recognizing this epitope.

Immune responses against Nef126-10 epitopes

We compared the epitope-specific immune responses between two groups of individuals: those whose plasma viruses were 133I/135F (n = 4) or 133T/135F (n = 10). *Ex vivo* IFN-γ ELISpot assays using PBMCs and Nef126-10(8I10F) or Nef126-10(8T10F) revealed that 0 of 4 patients with Nef126-10(8I10F) viruses had Nef126-10 (8T10F)-specific responses (Figure 6B). Nine out of 10 patients with Nef126-10(8T10F) viruses exhibited specific

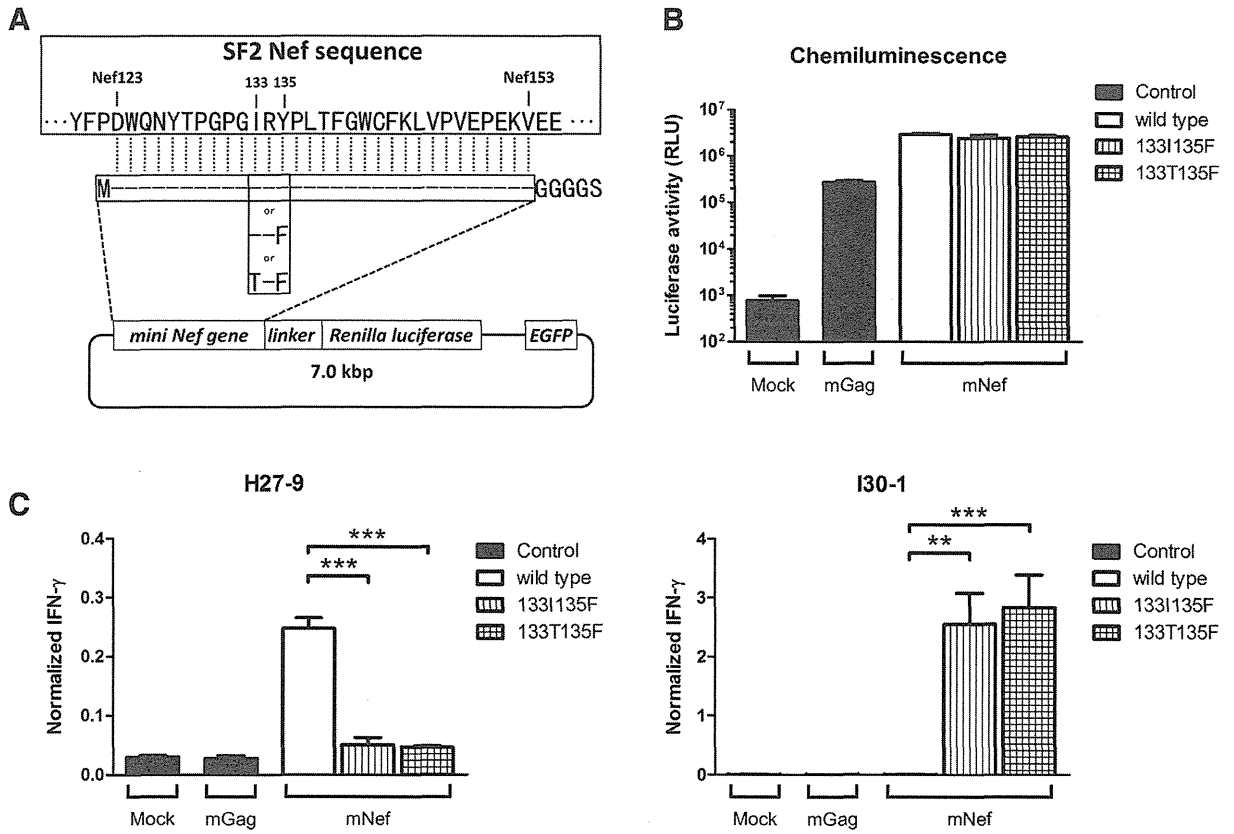

Figure 3 Recognition of endogenously derived epitope. (A) Vector construction. The vector expresses EGFP as a transfection marker and a fusion protein that consists of mini-Nef gene coding 31 amino acids of Nef123-Nef153 region and GlyGlyGlyGlySer linker and renilla luciferase. The 133I/135F and 133T/135F mutant vectors as well as wild type vector were constructed. Mini-Nef sequence that corresponds to the actual Nef gene is shown. **(B)** By measuring luciferase activity, quantity of the generated mini-Nef was assessed. No significant differences in mini-Nef generation between wild type, 135F and 133T/135F were observed. Each stick and bar indicates average and standard deviation of three independent experiments. **(C)** The vector transfected cells were incubated with either H27-9 or I30-1, and IFN-γ secretion was quantified by ELISA and normalized to determine the epitope recognition. For normalization, each value was divided by IFN-γ secretion with wild type peptide concentration of 9 μM. Mock as well as mGag, transfected with mGag(wt)-hRluc-EGFP, were measured as negative controls. Each stick and bar indicates average and standard deviation of three independent experiments. The significance was calculated by unpaired Student's t-test; **, $p < 0.01$; ***, $p < 0.001$.

responses to the circulating epitope, and 7 of 10 patients retained the response specific against Nef126-10(8I10F). These results strongly suggested that the I133T mutation induced a new subset of CD8+ T cells capable of recognizing Nef126-10(8T10F) ($p = 0.005$, Fisher's exact test).

Functional avidity has been reported as a correlate of CTL selective pressure [19,20]. As such, we analyzed functional avidities of Nef126-10-specific CTLs. Nine individuals harboring 133T/135F were analyzed by limiting dilution (Figure 6C). Nef126-10(8I10F)-specific CTL responses showed significantly higher avidities compared to those against Nef126-10(8T10F). Taken together with the observation that pVL correlated inversely with the magnitude of Nef126-10(8I10F)-specific, but not Nef126-10(8T10F)-specific, responses (Figure 5C), these results suggest that the new subset of CD8+ T cells elicited following selection of I133T exert less immune pressure on the 133T mutant compared to the "wild-type" I133. The hypothesis that Nef-I133T is an A*24-driven escape mutation is additionally supported by numerous HLA-association studies in HIV subtype B-infected populations including Japan, which consistently demonstrate highly significant associations between A*24 and Nef-I133T [4,8,21,22].

Discussion

HLA-A*24:02 is highly prevalent among East Asians including Japanese [11]. In an effort to identify immunodominant CTL epitopes presented by HLA-A*24:02, we observed that the two most frequently-recognized epitopes lay in Nef and overlapped each other by two amino acids. Nef codon 135 is critical to both epitopes, as it represents the N-terminal anchor for the downstream epitope Nef134-10, and the C-terminal anchor

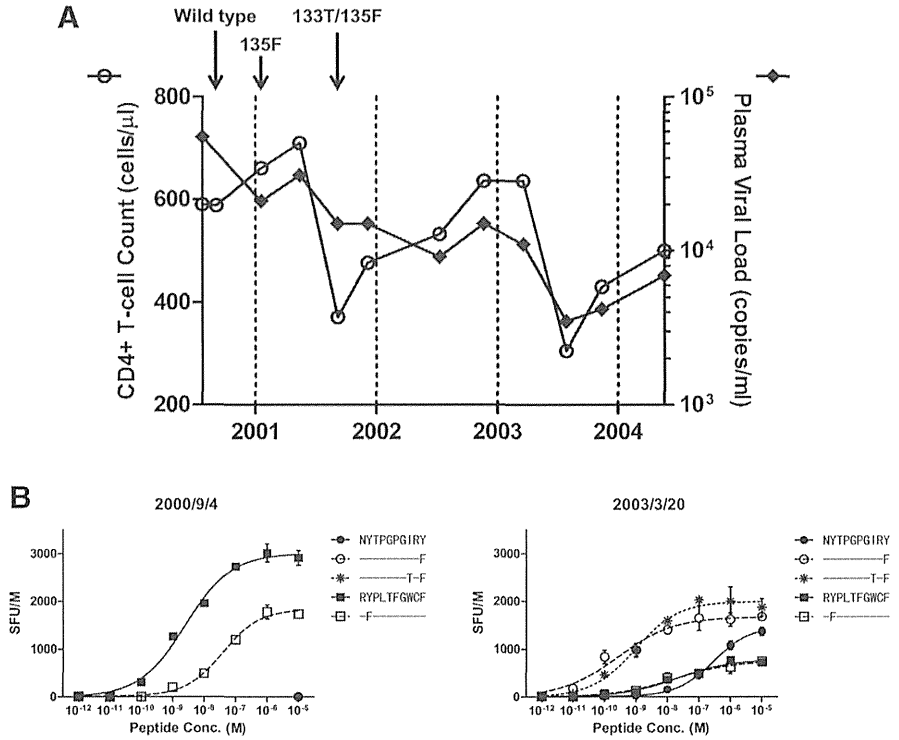

Figure 4 Longitudinal analysis in one individual. (A) Time courses of the surrogate markers are shown. CD4+ T cell count (left axis, white circle) and pVL (right axis, black circle) are plotted on the chart. Each arrow indicates the earliest time point when the sequence above the arrow was observed. **(B)** IFN-γ ELISpot assay was performed by using serially diluted peptide set and expanded PBMCs. Two time points were tested for the assay. At the earlier point, 2000/9/4, the patient harbored wild type sequence around the Nef126-10/Nef134-10 region, and at the latter point, 2003/3/20, the sequence displayed 133T/135F. Mutants as well as wild type peptides of Nef126-10 or Nef134-10 epitopes were used in the assay. Each point represents the average SFU of duplicate wells after subtraction of background, and each error bar shows standard deviation.

for the upstream epitope Nef126-10. In the downstream epitope Nef134-10, the Y-to-F mutation (Y135F) at the second position is observed at high frequencies in circulating HIV-1 sequences in Japan – in fact it represents the consensus at this position in Japan - presumably as a result of high HLA-A*24:02 prevalence in the population [4,23]. Our experiments using a Nef134-10-specific CTL clone and a minigene corroborated the earlier observation that the Y135F mutation disrupts antigen processing of the Nef134-10 epitope (Figure 3C) [4,12]. Importantly, while the majority of patients with Y135F responded to the upstream epitope Nef126-10, none of the patients with the wild-type sequence responded to this epitope. Consistent with this observation, results of the peptide binding (Figure 2A) and limiting dilution experiments using antigen-specific CTLs (Figure 2B) were compatible with the previous reports indicating that F, but not Y, could serve as a C-terminal anchor [16,17]. Also consistent with this observation is that the 2nd position of Nef126-10 is Y, a strong N-terminal anchor amino acid for HLA-A*24:02. Taken together, in a process similar to the ongoing exposure of novel antibody epitopes in HIV-1 envelope as a consequence of escape from earlier humoral responses [10], our results demonstrate that an analogous phenomenon also occurs with CTL responses: in this case a novel A*24:02-restricted "epitope switch" from Nef134-10 to Nef126-10, as a result of immune-driven escape at a single Nef codon.

We also showed that Nef residues I133T and Y135F are highly significantly linked *in vivo*. Nef126-10 emerges as a CTL epitope by the introduction of the Y135F mutation. Though I133T has previously been identified as an HLA-A*24:02-associated polymorphism in statistical association studies [4,8,21,22,24,25], its mechanism remained unknown. Our data strongly suggest that I133T is HLA-A*24:02-restricted escape mutation whose mechanism of action is alteration of the *in vivo* CTL repertoire capable of recognizing the HLA-bound epitope. Although the sample size was limited, patients with 133I/135F viruses did not exhibit responses to Nef126-10(8T10F) (Figure 6B). These results, together with studies of a patient whose plasma viral sequences shifted from wild-type to 133T/135F, strongly suggest that immune pressures selected an I-to-T substitution at Nef's 133rd position. IFN-γ ELISpot assays showed that Nef126-10(8I10F)-specific but not Nef126-10(8T10F)-specific responses

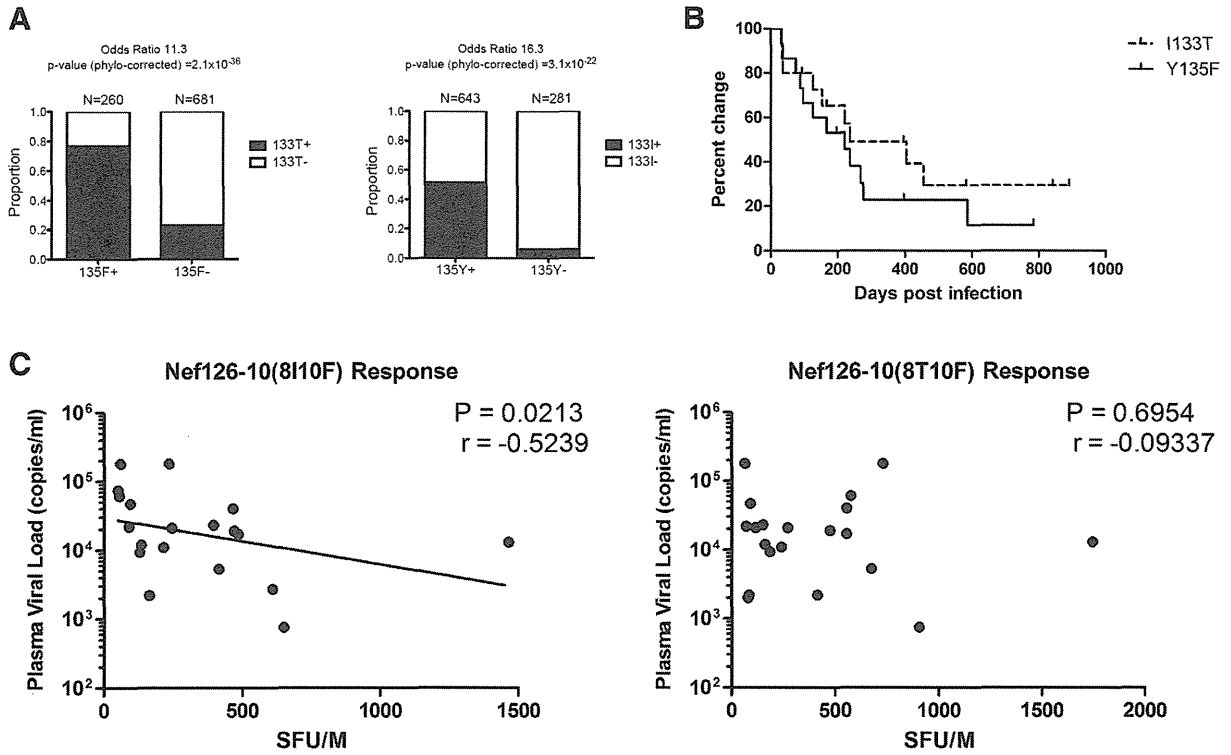

Figure 5 Relevance of I133T mutation to Y135F and its impact on CTL responses. (A) The cross-sectional analysis of the co-variation relationship between Nef codons 133 and 135. Nef codons containing gaps or amino acid mixtures were excluded from analysis, leaving 941 and 924 sequences for analysis of codon pairs 135F/133T and 135Y/133I, respectively. **(B)** Kaplan-Meier plot of the emergence of Y135F and I133T mutations in a multicenter longitudinal acute/early infection cohort comprising 16 HLA-A*24:02-expressing persons. **(C)** Within 24 patients who harboring 133I/135F or 133T/135F mutant, correlation between the magnitude of peptide-specific response and pVL was examined. Peptide-specific response was determined by using *ex vivo* IFN-γ ELISpot assay with frozen PBMCs. Each point is plotted according to the average SFU of duplicate wells and the measured pVL at the sampling point. Statistical significance of the correlation between the magnitude and pVL was calculated by Spearman's rank correlation.

correlated inversely with pVL (Figure 5C) and that the former had significantly higher functional avidities (Figure 6C). These findings therefore suggest a TCR-mediated mechanism underlying HLA-A*24:02-mediated escape via I133T. Although higher functional avidity is a hallmark of CTLs with stronger selective pressure [19,20], further studies are needed to confirm that the I133T mutation alleviates immune pressures directed on the Nef126-10 epitope.

Crystal structures of peptide-HLA showed that the side chain of the 133rd residue (P8 residue in the Nef126-10 epitope) protruded from the peptide-binding cleft presumably providing a feature of the Nef126-10 epitope to the TCRs (Figure 6A). The shorter side chain of T compared to I might make the Nef126-10(8T10F) less accessible to TCR than the Nef126-10(8I10F) epitope. Considering the similarity of the structures, the absence of the T cell repertoire against the Nef126-10(8T10F) epitope in the patients with 133I/135F viruses is an enigma. The suggested structural flexibility of the central portion (P5-P7) and P9 of the Nef126-10 epitope may be relevant here.

A key remaining question is why the Y135F mutation is repeatedly selected by A*24:02, given that a consequence of this escape is the introduction of another A*24:02 epitope immediately upstream. We offer the following hypothesis. In studies of HIV-1 infected populations around the globe, the association between HLA-A*24:02 and Nef-135F consistently ranks among the strongest in the HIV proteome [8,21], including in Japan where F (rather than the global subtype B consensus Y) represents the consensus at this position [4,22]. Indeed, a recent international cohort study revealed an odds ratio of >28 and a p-value of $8x10^{-118}$ for this association [21]. The extraordinary magnitude of this association indicates that Nef-135 is under similarly extraordinary selection pressure by A*24 *in vivo* - presumably due to highly effective CTL responses against the Nef134–10 epitope. The benefits to HIV of evading A*24-mediated recognition of Nef134–10 presumably outweigh its substantial negative consequences to the virus, which in this case include the creation of the adjacent Nef126-10 epitope. That Nef126-10 is targeted by less than 70% of A*24-expressing