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Identification of SIV Nef CD8$^+$ T cell epitopes restricted by a MHC class I haplotype associated with lower viral loads in a macaque AIDS model

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ABSTRACT

Virus-specific CD8$^+$ T-cell responses are crucial for the control of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) replication. Multiple studies on HIV-infected individuals and SIV-infected macaques have indicated association of several major histocompatibility complex class I (MHC-I) genotypes with lower viral loads and delayed AIDS progression. Understanding of the viral control mechanism associated with these MHC-I genotypes would contribute to the development of intervention strategy for HIV control. We have previously reported a rhesus MHC-I haplotype, *90-120-Ia*, associated with lower viral loads after SIVmac239 infection. $Gag_{206–216}$ and $Gag_{241–249}$ epitope-specific CD8$^+$ T-cell responses have been shown to play a central role in the reduction of viral loads, whereas the effect of Nef-specific CD8$^+$ T-cell responses induced in all the *90-120-Ia*$^+$ macaques on SIV replication remains unknown. Here, we identified three CD8$^+$ T-cell epitopes, $Nef_{9–19}$, $Nef_{89–97}$, and $Nef_{193–203}$, associated with *90-120-Ia*. $Nef_{9–19}$ and $Nef_{193–203}$ epitope-specific CD8$^+$ T-cell responses frequently selected for mutations resulting in viral escape from recognition by these CD8$^+$ T cells, indicating that these CD8$^+$ T cells exert strong suppressive pressure on SIV replication. Results would be useful for elucidation of the viral control mechanism associated with *90-120-Ia*.

1. Introduction

In human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) infections, host immune responses fail to eradicate viruses and allow persistent infection, leading to AIDS progression. Unlike most acute virus infections, effective neutralizing antibody responses are not efficiently induced in early HIV/SIV infection [1]. Virus-specific CD8$^+$ T-cell responses play an important role in the control of HIV/SIV replication [2–6]. CD8$^+$ T cells recognize antigenic peptides bound to polymorphic major histocompatibility complex class I (MHC-I) molecules, whose genotypes affect CD8$^+$ T-cell responses [7,8]. Several MHC-I genotypes have been shown to be associated with lower viral loads and slower disease progression in HIV/SIV infections [9–14]. Understanding of the viral control mechanism associated with these protective MHC-I alleles would contribute to the development of intervention strategy for HIV control.

Recent vaccine trials in macaque AIDS models have shown a possibility of SIV control by effective CD8$^+$ T-cell responses [15–19]. It has been indicated that CD8$^+$ T cells targeting Gag are effective against HIV/SIV infection [20–23]. Furthermore, current studies have suggested that Nef- and Vif-specific CD8$^+$ T-cell responses can contribute to SIV control in macaque AIDS models [24,25].

We have previously reported a rhesus MHC-I haplotype, *90-120-Ia*, associated with lower viral loads after SIVmac239 challenge [14]. In that study, those Burmese rhesus macaques possessing *90-120-Ia* had lower set-point plasma viral loads (geometric mean at 1 year after SIV challenge: 1.5×10^4 copies/ml); two of them controlled viremia for more than 4 years while the remaining four developed AIDS in 4 years. Our vaccine trial has shown that all the *90-120-Ia*$^+$ macaques immunized with DNA-prime/Gag-expressing Sendai virus (SeV-Gag) vector-boost controlled a SIVmac239 challenge [26]. Mamu-A1*043:01-restricted $Gag_{206–216}$ (IINEEAADWDL) and Mamu-A1*065:01-restricted $Gag_{241–249}$ (SSVDEQIQW) epitope-specific CD8$^+$ T-cell responses were responsible for this viral control

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[26–28]. SIVmac239-infected *90-120-Ia*$^+$ macaques also elicited CD8$^+$ T-cell responses targeting Nef, which may be involved in viral control [14]. In the present study, we determined Nef CD8$^+$ T-cell epitopes associated with this MHC-I haplotype *90-120-Ia*.

2. Materials and methods

2.1. Samples

The present study used frozen plasma and peripheral mononuclear cell (PBMC) samples derived from ten Burmese rhesus macaques (*Macaca mulatta*) possessing MHC-I haplotype *90-120-Ia*. Our previous SIVmac239 challenge experiments using these animals [14,26–28] have been carried out in Tsukuba Primate Research Center in National Institute of Biomedical Innovation (NIBP) with the help of the Corporation for Production and Research of Laboratory Primates. These studies were approved by the Committee on the Ethics of Animal Experiments of NIBP under the guideline for animal experiments at NIBP 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).

Macaques R06-037, R07-004, and R07-009 were unvaccinated and intravenously challenged with SIVmac239 [14]. Macaques R03-018 and R07-007 received a DNA-prime/SeV-boost vaccine eliciting $Gag_{206–216}$- and $Gag_{241–249}$-specific CD8$^+$ T-cell responses, respectively, before SIVmac239 challenge as described before [27,28]. Macaques R06-035, R06-041, R05-004, R05-027, and R07-005 received a DNA-prime/SeV-Gag-boost as described before [26]. Macaques R06-035 and R06-041 were intravenously challenged with SIVmac239Gag216S244E, a SIVmac239 carrying two *gag* mutations, GagL216S and GagD244E, leading to a leucine (L)-to-serine (S) substitution at the 216th amino acid (aa) and an aspartic acid (D)-to-glutamic acid (E) substitution at the 244th aa in Gag, whereas macaques R05-004, R05-027, and R07-005 with SIVmac239Gag216S244E247L312V373T, a SIVmac239 carrying five *gag* mutations, GagL216S, GagD244E, GagI247L (isoleucine [I] to L at the 247th aa), GagA312V (alanine [A] to valine [V] at the 312th aa), and GagA373T (A to threonine [T] at the 373rd aa) [26].

The determination of macaque MHC-I haplotypes was based on the family study in combination with the reference strand-mediated conformation analysis of *Mamu-A* and *Mamu-B* genes and detection of major *Mamu-A* and *Mamu-B* alleles by cloning the reverse transcription (RT)-PCR products [14,29,30]. Confirmed MHC-I alleles consisting of the MHC-I haplotype *90-120-Ia* are *Mamu-A1*043:01* (GenBank accession number AB444869), *Mamu-A1*065:01* (AB444921), *Mamu-B*061:03* (AB430442), *Mamu-B*068:04* (AM902571), and *Mamu-B*089:01* (EF580172).

2.2. Sequencing analysis of plasma viral genomes

Viral RNAs were extracted using the High Pure Viral RNA kit (Roche Diagnostics) from plasma. Fragments of cDNAs encoding SIVmac239 Nef were amplified by nested RT-PCR from plasma RNAs and subjected to direct sequencing by using dye terminator chemistry and an automated DNA sequencer (Applied Biosystems) [33].

2.3. Analysis of SIV peptide-specific CD8$^+$ T-cell responses

SIV peptide-specific CD8$^+$ T-cell responses were measured by flow-cytometric analysis of interferon-γ (IFN-γ) induction [25]. PBMCs (2.5×10^6 cells) were cocultured for 6 h with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines (B-LCLs; 1.0×10^6 cells) pulsed with 1–5 μM or indicated concentrations of peptides designed for epitope mapping in 96-well V-bottom microwell plates. Intracellular IFN-γ staining was performed using a Cytofix Cytoperm kit (BD). Fluorescein isothiocyanate-conjugated anti-human CD4 (BD), peridinin chlorophyll protein (PerCP)-conjugated anti-human CD8 (BD), allophycocyanin Cy7 (APC-Cy7)-conjugated anti-human CD3 (BD), and phycoerythrin (PE)-conjugated anti-human IFN-γ antibodies (Biolegend) were used. Specific T-cell frequencies were calculated by subtracting nonspecific IFN-γ$^+$ T-cell frequencies from those after peptide-specific stimulation. Specific T-cell frequencies less than 100 cells per million PBMCs were considered negative.

3. Results and discussion

3.1. Identification of three Nef CD8$^+$ T-cell epitopes associated with MHC-I haplotype 90–120-Ia

In our previous study [14], we examined viral genome sequences 1 year after SIVmac239 challenge in four groups of Burmese rhesus macaques possessing MHC-I haplotypes *90-120-Ia*, *90-120-Ib*, *90-010-Ie*, and *90-088-Ij*, respectively. Then, in the present study, we compared *nef* sequences in the four macaques possessing *90-120-Ia* with those in the remaining three groups ($n = 14$). Amino acid sequences revealed three regions in Nef, Nef12 (the 12th aa), Nef89/90 (the 89th or 90th aa), and Nef201/202 (the 201st or 202nd aa), which had substitutions in all *90-120-Ia*$^+$ animals but mostly not in others. Indeed, substitutions at Nef12 were observed only in two of the fourteen *90-120-Ia*-negative animals while substitutions at Nef89/90 or Nef201/202 were detected in none of them.

We tried to map CD8$^+$ T-cell epitopes around the regions described above to examine whether these *90-120-Ia*-associated *nef* mutations resulting in the Nef12, Nef89/90, and Nef201/202 amino acid substitutions were selected by CD8$^+$ T cells. Analysis using available samples derived from ten *90-120-Ia*$^+$ macaques identified three CD8$^+$ T-cell epitopes, $Nef_{9–19}$ (RSRPSGDLRQR), $Nef_{89–97}$ (DIDEEDDDL), and $Nef_{193–203}$ (YLMHPAQTSQW) (Fig. 1A). The endpoint peptide concentrations for CD8$^+$ T-cell responses were 10–100 nM against $Nef_{9–19}$ epitope and 1–10 nM against $Nef_{193–203}$ (Fig. 1B). The endpoint was very low, less than 0.1 nM, for $Nef_{89–97}$ epitope (Fig. 1B), indicating extremely high binding affinity of this epitope.

3.2. Determination of MHC-I alleles restricting CD8$^+$ T-cell epitopes

$Nef_{9–19}$ epitope-specific CD8$^+$ T-cell responses in the early phase of SIV infection were examined in six *90-120-Ia*$^+$ animals, all of which showed positive responses (Fig. 2A), indicating that this epitope is associated with MHC-I haplotype *90-120-Ia*. On the other hand, $Nef_{89–97}$ and $Nef_{193–203}$ epitope-specific CD8$^+$ T-cell responses in the early phase were detected not in all but in three of the seven and two of the four examined *90-120-Ia*$^+$ animals, respectively (Fig. 2A).

We then tried to determine *90-120-Ia*-derived MHC-I alleles restricting these CD8$^+$ T-cell epitopes. HLA-A/B/C-negative human 721.221 cell lines expressing *Mamu-A1*043:01*, *Mamu-A1*065:01*, and *Mamu-B*061:03* were available for the analysis. $Nef_{89–97}$-specific CD8$^+$ T-cell responses were detected on *Mamu-A1*043:01*-expressing 721.221 cells, whereas $Nef_{193–203}$-specific CD8$^+$ T-cell responses were detected on *Mamu-A1*065:01*-expressing 721.221 cells (Fig. 2B). These results indicate that the $Nef_{89–97}$ and $Nef_{193–203}$ epitopes are restricted by Mamu-A1*043:01 and Mamu-A1*065:01, respectively. However, $Nef_{9–19}$ epitope-specific CD8$^+$ T-cell responses were not detected on any of 721.221 cells expressing *Mamu-A1*043:01*, *Mamu-A1*065:01*, or *Mamu-B*061:03*,

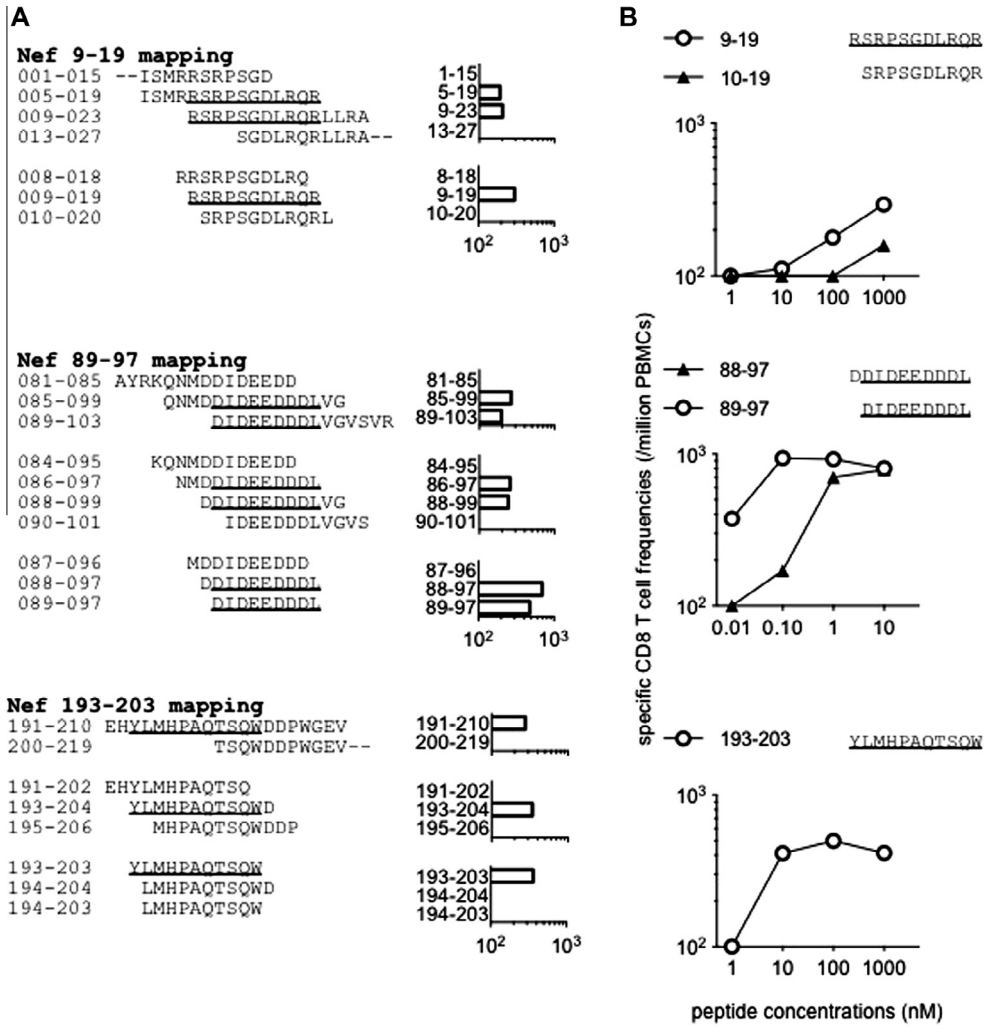


Fig. 1. Mapping of CD8$^+$ T-cell epitopes, Nef_{9-19}, Nef_{89-97}, and $Nef_{193-203}$. (A) Summarized data for mapping of Nef_{9-19} (top panels), Nef_{89-97} (middle), and $Nef_{193-203}$ (bottom) epitopes using PBMCs of SIV-infected *90-120-Ia*$^+$ animals. CD8$^+$ T-cell frequencies specific for the indicated peptides are shown (/million PBMCs). Representative results using PBMCs from R05-027 and R06-041 (top), R03-018 (middle), and R07-004 and R07-007 (bottom) are shown. (B) CD8$^+$ T-cell responses under the indicated concentrations of Nef_{9-19} and Nef_{10-19} (top panel), Nef_{88-97} and Nef_{89-97} (middle), and $Nef_{193-203}$ (bottom) peptides. Representative results using PBMCs from R05-004 (top), R03-018 (middle), and R07-009 (bottom) are shown.

implying that this epitope is restricted by a *90-120-Ia*-derived MHC-I molecule other than the above three (Fig. 2B).

3.3. Mutations resulting in viral escape from CD8$^+$ T-cell recognition

In our previous study [14], SIV-infected *90-120-Ia*$^+$ macaques had mutations resulting in Nef12, Nef89/90, and Nef201/202 amino acid substitutions as described above. The substituted amino acids were different at Nef89/90 in individual four animals, but three of the four had the same substitutions at Nef12 and Nef201, NefP12Q (proline [P]-to-glutamine [Q]) and NefS201Y (S-to-tyrosine [Y]), respectively. We examined whether these two *90-120-Ia*-associated *nef* mutations result in viral escape from CD8$^+$ T-cell responses specific for the epitopes we identified. Nef_{9-19} peptide-specific CD8$^+$ T-cell responses were reduced by the NefP12Q substitution (Fig. 3A). Also, the NefP12T substitution (a P-to-T substitution at the 12th aa in Nef) that was observed in the remaining one SIV-infected *90-120-Ia*$^+$ macaque resulted in viral escape from Nef_{9-19}-specific CD8$^+$ T-cell responses. $Nef_{193-203}$ peptide-specific CD8$^+$ T-cell responses were reduced by the NefS201Y (Fig. 3A). Selection of these escape mutations in SIV infection implies that these Nef_{9-19} and $Nef_{193-203}$ epitope-specific CD8$^+$ T cells exert suppressive pressure on SIV replication. The latter $Nef_{193-203}$ epitope overlaps with previously-reported MW9 ($Nef_{195-203}$) and HW8 ($Nef_{196-203}$) epitopes [32,33]. The MW9 is restricted by Mamu-B*17 [12], a protective MHC-I against SIVmac239 infection, while the HW8 is restricted by a MHC-I in a group of Mauritian cynomolgus macaques that frequently control SIVmac239 replication. Thus, this $Nef_{193-203}$ region may be a promising CD8$^+$ T-cell target for SIV control.

Further analysis of viral *nef* nucleotide sequences found relatively rapid selection of a mutation encoding Nef12, NefP12Q, NefP12S, or NefS13P, in two months after SIV infection in macaques R05-004, R05-027, R06-035, R06-041, and R07-005 (Fig. 3B). The NefP12S and NefS13P substitutions also resulted in viral escape from Nef_{9-19}-specific CD8$^+$ T-cell responses (Fig. 3A). However, no mutation was selected in the region encoding Nef_{89-97} or $Nef_{193-203}$ epitope in two months (Fig. 3B). In the early phase, Nef_{9-19}-specific CD8$^+$ T-cell responses were induced in all whereas Nef_{89-97}- and $Nef_{193-203}$-specific CD8$^+$ T-cell responses were detectable only in some of them, as described above. These results suggest that *90-120-Ia*$^+$ macaques predominantly elicit Nef_{9-19}-specific CD8$^+$ T-cell responses resulting in selection of Nef12/13 mutations in the early phase of SIV infection, followed by induction of Nef_{89-97}- and $Nef_{193-203}$-specific CD8$^+$ T-cell responses resulting in selection of Nef89/90 and Nef201/202 mutations in the chronic phase.

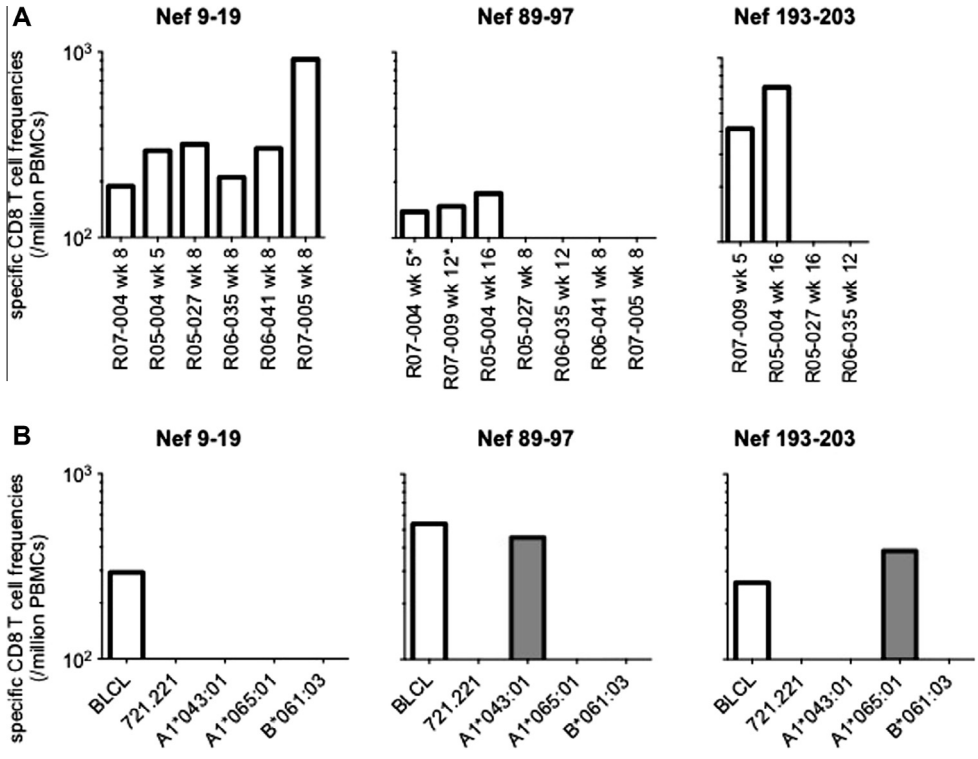
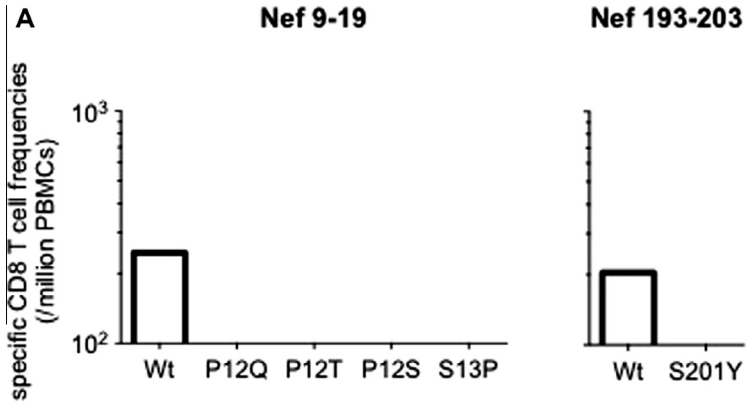


Fig. 2. Nef_{9-19}, Nef_{89-97}, and $Nef_{193-203}$ epitope-specific $CD8^+$ T-cell responses. (A) $CD8^+$ T-cell responses specific for Nef_{9-19} (left panel), Nef_{89-97} (middle), and $Nef_{193-203}$ (right) epitopes in the early phase of SIV infection in *90-120-Ia*$^+$ macaques. The asterisk indicates $CD8^+$ T-cell responses specific for Nef_{86-97} peptide. (B) Nef_{9-19}- (left panel), Nef_{89-97}- (middle), and $Nef_{193-203}$-specific (right) $CD8^+$ T-cell responses after coculture with peptide-pulsed B-LCLs, 721.221 cells, or 721.221 cells expressing *Mamu-A1*043:01*, *Mamu-A1*065:01*, or *Mamu-B*061:03*. These cells were pulsed with 1,000 nM Nef_{9-19} peptides (left), 1 nM Nef_{89-97} peptides (middle), and 100 nM $Nef_{193-203}$ peptides (right), respectively. Representative results using PBMCs from R06-035 for Nef_{9-19}, R03-018 for Nef_{89-97}, and R07-004 for $Nef_{193-203}$ are shown.



B

Nef	9-19	89-97	193-203
	RSRPSGDLRQR	DIDEEDDDL	YLMHPAQTSQW
R05-004	---S-------	---------	----------
R05-027	---Q-------	---------	----------
R06-035	---SP------	---------	----------
R06-041	---S-------	---------	----------
R07-005	----P------	---------	----------G

Fig. 3. $CD8^+$ T-cell escape mutations. (A) $CD8^+$ T-cell responses specific for the wild-type or mutant Nef_{9-19} peptides with the indicated substitutions (left panel) or the wild-type or a mutant $Nef_{193-203}$ peptide with S201Y substitution (right). Representative results using PBMCs from R07-005 for Nef_{9-19} and R07-004 for $Nef_{193-203}$ are shown. (B) Predominant nonsynonymous mutations in plasma viral *nef* regions encoding Nef_{9-19}, Nef_{89-97}, and $Nef_{193-203}$ epitopes in 3 months after SIV challenge in *90-120-Ia*$^+$ macaques. Amino acid substitutions are shown.

Our previous study [14] frequently found $CD8^+$ T-cell responses targeting Vif and a viral genome mutation resulting in VifP115S substitution (a P-to-S substitution at the 115th aa in Vif) in SIV-infected *90-120-Ia*$^+$ macaques. Then, in the present study, we identified a $CD8^+$ T-cell epitope, $Vif_{114-124}$ (FPCFTAGEVRR). The endpoint peptide concentration for $Vif_{114-124}$-specific $CD8^+$ T-cell

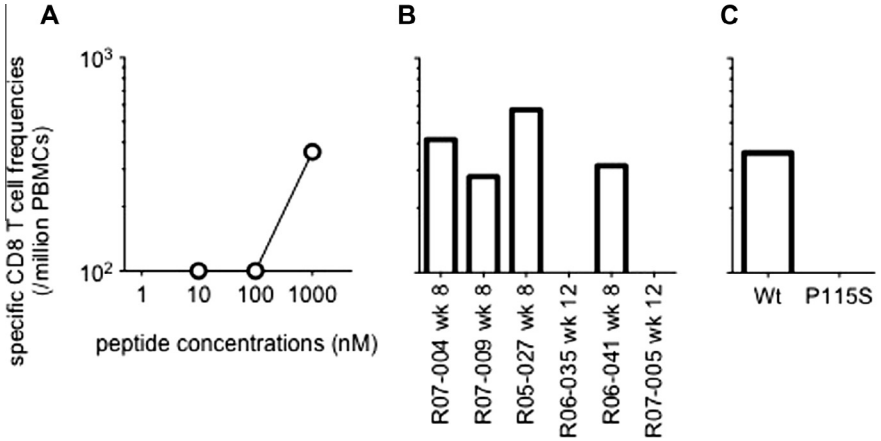


Fig. 4. Characterization of $Vif_{114–124}$ epitope-specific $CD8^+$ T-cell responses. (A) $CD8^+$ T-cell responses under the indicated concentrations of $Vif_{114–124}$ peptides. A representative result using PBMCs from R07-004 is shown. (B) $Vif_{114–124}$-specific $CD8^+$ T-cell responses in the early phase of SIV infection in *90-120-Ia*$^+$ macaques. (C) $CD8^+$ T-cell responses specific for the wild-type or a mutant $Vif_{114–124}$ peptide with P115S substitution. A representative result using PBMCs from R07-004 is shown.

responses was very high, more than 100 nM, indicating lower binding affinity of this epitope (Fig. 4A). $Vif_{114–124}$ epitope-specific $CD8^+$ T-cell responses were detected in the early phase in four of the six examined *90-120-Ia*$^+$ animals (Fig. 4B). The VifP115S substitution resulted in diminishment of $Vif_{114–124}$ peptide-specific $CD8^+$ T-cell responses, suggesting selective pressure by $CD8^+$ T cells targeting this epitope (Fig. 4C).

In summary, we identified three *90-120-Ia*-associated Nef $CD8^+$ T-cell epitopes, $Nef_{9–19}$, $Nef_{89–97}$, and $Nef_{193–203}$, in addition to the three previously-identified Gag epitopes, $Gag_{206–216}$, $Gag_{241–249}$, and $Gag_{373–380}$ [31]. Additionally, we identified a Vif $CD8^+$ T-cell epitope, $Vif_{114–124}$. In our previous study [26], all the *90-120-Ia*$^+$ macaques vaccinated with DNA-prime/SeV-Gag-boost controlled SIVmac239 replication without detectable viral loads after week 5 post-challenge, whereas those vaccinated animals (R05-004, R05-027, R06-035, R06-041, and R07-005) failed to show such rapid control of a challenge with SIVs carrying $Gag_{206–216}$-, $Gag_{241–249}$-, and $Gag_{373–380}$-specific $CD8^+$ T-cell escape mutations. This indicates that these $Gag_{206–216}$, $Gag_{241–249}$, and $Gag_{373–380}$ epitope-specific $CD8^+$ T-cell responses are responsible for the rapid SIVmac239 control. Macaques R05-004 and R05-027 showed persistent viremia and developed AIDS, whereas the remaining three (R06-035, R06-041, and R07-005) exhibited lower viral loads. The present study suggests involvement of Nef epitope-specific $CD8^+$ T-cell responses in this suppression of SIV replication in *90-120-Ia*$^+$ macaques.

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