inverse correlation between such effector functions and viral loads in HIV-infected individuals [5,6] and vaccinated SIV-infected macaques [11–14], the precise influence of non-NAb responses on viral replication control remains undetermined. Passive immunization studies in nonhuman primate AIDS models have shown partial protection from mucosal virus challenge by mucosal pre-challenge non-NAb infusion, suggesting limited protective efficacy of locally-distributed non-NAb responses [15,16]. In the present study, we focused on the effect of systemic distribution of non-NAbs on established primary viral infection, which is another practical vaccine correlate.

Passive immunization of polyclonal neutralizing antibodies (NAbs), which does not exclude coexistence of non-NAbs, has partially provided protective activity in nonhuman primate AIDS models [17–19]. Additionally, we have reported SIV control *in vivo* by post-infection administration of polyclonal NAbs, in which enhanced antigen presentation and subsequent augmented T-cell responses likely accounted for the control [20,21]. Since non-NAbs are potentially capable of supporting these suggested mechanisms, the protective activity of non-NAbs by themselves against established primary infection is important to be assessed. Here, we examined the effect of passive non-NAb immunization at day 7 post-challenge on primary SIV_{mac239} replication in rhesus macaques. Despite the virion-binding and ADCVI activity of non-NAbs having been confirmed *in vitro*, passive immunization of non-NAbs did not result in control of SIV replication *in vivo*.

Methods

Ethics Statement

Animal experiments were carried out in National Institute of Biomedical Innovation (NIBP) after approval by the Committee on the Ethics of Animal Experiments of NIBP in accordance with the guidelines for animal experiments at NIBP and National Institute of Infectious Diseases. To prevent viral transmission, animals were housed in individual cages allowing them to make sight and sound contact with one another, where the temperature was kept at 25 °C with light for 12 hours per day. Animals were fed with apples and commercial monkey diet (Type CMK-2, Clea Japan, Inc.). Blood collection, vaccination, and SIV challenge were performed under ketamine anesthesia. Three of eleven macaques, R10-001, R10-004, and R06-029, were euthanized during the observation period in the SIV challenge experiment of this study. Two of them (R10-004 and R06-029) were euthanized (at 7-10 months) after the minimum observation period required for this study (6 months) because of the limitation of available cage numbers. One macaque R10-001 was euthanized (at 9 months) at the endpoint for euthanasia, which was determined by typical signs of AIDS including reduction in peripheral $CD4^+$ T-cell counts (less than 200 cells/μl), 10% loss of body weight, diarrhea, and general weakness. At euthanasia, animals were deeply anesthetized with pentobarbital under ketamine anesthesia, and then, whole blood was collected from left ventricle.

Analysis of Virus-Specific Neutralizing Responses

Heat-inactivated plasma or purified antibodies were prepared in quadruplicate and mixed with 10 $TCID_{50}$ (50 percent tissue culture infective dose) of SIV_{mac239} [22]. In each mixture, 5 μl of diluted sample was incubated with 5 μl of virus. After 45 min incubation at room temperature, each 10 μl mixture was added into 5 x 10^4 HSC-F cells (macaque T cell line) [23] per well in 96-well plates. Day 10 culture supernatants were harvested and progeny virus production was examined by determining the supernatant reverse transcriptase activity to confirm the absence of neutralizing activity at 1:2.

Whole virus ELISA and immunoblotting. SIV virions used for the antigen were prepared by infecting HSC-F cells with SIV_{mac239} at MOI 0.01. Day 7 supernatant was collected and virus particles were purified by centrifugation at 35,000 rpm, 75 min on 20% sucrose in a SW41 rotor (Beckman Coulter), followed by 35,000 rpm, 75 min on 20%-60% sucrose in a SW55 rotor (Beckman Coulter) and 35,000 rpm, 75 min on 20% sucrose in a SW41 rotor. Precipitated SIV virions were diluted in phosphate buffered saline (PBS) and used to coat 96-Well Assay Plates (Becton Dickinson) at a concentration of 100 ng/ml p27 (0.1 ml per well) by overnight incubation at 4 °C. Wells were washed with PBS and blocked with 0.5% bovine serum albumin (BSA)/PBS. Purified anti-SIV immunoglobulin G (IgG) serially diluted in PBS (0.1 ml per well) were incubated for 2 hr at 37 °C. Plates were washed with PBS and virion-bound antibodies were detected with a horseradish peroxidase (HRP)-conjugated goat anti-monkey IgG (H+L) (Bethyl Laboratory) and SureBlue TMB 1-Component Microwell Peroxidase Substrate (KPL). SIV-specific IgG activity in the purified IgG and plasma samples were detected using a Western blotting system for detection of SIV_{mac251} antigens (ZeptoMetrix) according to the manufacturer's instruction. Samples from animals (R02-006, R04-011, R04-014, and R06-005) with rapid AIDS progression showed lower antibody reactivity.

Antibody-dependent cell-mediated virus inhibition (ADCVI) assay. HSC-F cells (1 x 10^5) serving as MHC-mismatched targets were infected with SIV_{mac239} at MOI 0.001. After adsorption for 6 hr, cells were washed twice with medium and serially-diluted anti-SIV or control antibodies (1.0 or 0.1 mg/ml) were added to the target cells with 4 x 10^5 effector cells, rhesus peripheral blood mononuclear cells (PBMCs), at an E:T ratio of 4:1 in round-bottomed 96-well plates. Wells of target cells without antibodies or effector cells were set as negative controls. After 7 days of culture, supernatants were collected and measured for their Gag p27 concentrations by ELISA (ABL). The percentage of virus inhibition deriving from ADCVI was calculated as follows: % inhibition = 100x (1 - [p27p/p27c]); where p27p and p27c are the average p27 concentrations in wells with anti-SIV and control antibodies, respectively. Experiments were performed twice in duplicate.

Antibody preparation. Ten lots of IgG solutions were prepared from ten chronically SIV_{mac239}-infected rhesus macaques without detectable SIV_{mac239}-specific NAb responses, respectively. IgG was purified from the plasma after heat-inactivation and filtration by Protein G Sepharose 4 Fast Flow (Amersham) and concentrated by Amicon Ultra 4, MW50000

(Millipore) to 30 mg/ml. Purified IgG solutions were confirmed negative for SIV_{mac239}-specific neutralizing activity. Three lots prepared from three macaques (R06-007, R01-009, and R03-005) were mixed to obtain the IgG inoculums for passive non-NAb immunization. Five lots of IgG solutions were also prepared from five chronically SIV_{mac239}-infected rhesus macaques with detectable SIV_{mac239}-specific NAb responses, respectively. Control IgG (CAb) was prepared from pooled plasma of non-infected rhesus macaques.

Animal experiments. Burmese rhesus macaques (*Macaca mulatta*) were challenged intravenously with 1,000 $TCID_{50}$ of SIV_{mac239}. For passive immunization, animals were intravenously administered with 300 mg of anti-SIV non-NAb IgG or control IgG at day 7 post-challenge. The determination of major histocompatibility complex class I (MHC-I) haplotypes was based on the family study in combination with the reference strand-mediated conformation analysis (RSCA) 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 as described previously [24–27]. Data on control macaques R10-005, R10-008, and R10-001 have previously been reported [28].

Measurement of virus-specific T-cell responses. Virus-specific $CD8^+$ T-cell responses were measured by flow-cytometric analysis of gamma interferon (IFN-γ) induction as described previously [29]. PBMCs were cocultured with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines (B-LCLs) pulsed with overlapping peptide pools spanning the SIV_{mac239} Gag, Pol, Vif, Vpx, Vpr, Tat, Rev, Env, and Nef amino acid sequence. Intracellular IFN-γ staining was performed using CytofixCytoperm kit (Becton Dickinson). Fluorescein isothiocianate-conjugated anti-human CD4, Peridinin chlorophyll protein-conjugated anti-human CD8, allophycocyanin-conjugated anti-human CD3 and phycoerythrin-conjugated anti-human IFN-γ antibodies (Becton Dickinson) were used. Specific T-cell levels were calculated by subtracting non-specific $IFN\text{-}\gamma^+$ T-cell frequencies from those after SIV-specific stimulation. Specific T-cell levels less than 100 cells per million PBMCs are considered negative.

Sequencing. Viral RNAs were extracted using High Pure Viral RNA kit (Roche Diagnostics) from macaque plasma obtained at around 1 year after challenge. Fragments of cDNAs encoding SIV_{mac239} Env 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). Predominant non-synonymous mutations were determined.

Statistical analysis. Statistical analysis was performed by Prism software version 4.03 (GraphPad Software, Inc.). Comparison of viral loads, peripheral blood $CD4^+$ T-cell counts, peripheral blood central memory $CD4^+$ T-cell frequencies, and the number of non-synonymous mutations in Env-coding regions between non-NAb-infused and control animals was performed by nonparametric Mann–Whitney U test with significance levels set at $p < 0.05$.

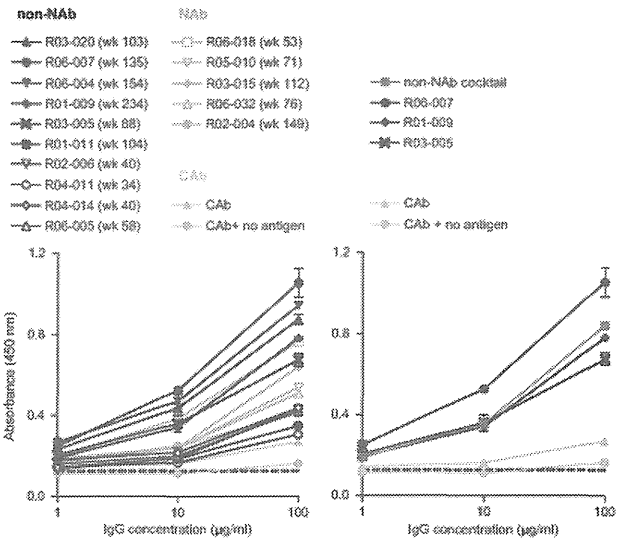

Figure 1. Binding properties of IgGs to SIV virions. Polyclonal IgGs purified from macaque plasma were subjected to whole virus ELISA using purified SIV_{mac239} virions as the antigen. Results on ten IgG lots derived from ten macaques without detectable neutralizing activity (non-NAbs; black lines), five with neutralizing activity (NAbs; red), and a control IgG (CAb; green) are shown in the left panel. Results on the non-NAb cocktail and three non-NAb lots composing the cocktail are in the right. The dotted line represents background absorbance. Time points of plasma sampling are shown in parentheses following the macaque IDs. A representative result, means and SDs of duplicate samples, from two experiments is shown.

doi: 10.1371/journal.pone.0073453.g001

Results

In vitro virion binding and ADCVI activity of SIV-specific non-NAbs

Ten lots of polyclonal IgG were prepared from plasma of ten chronically SIV_{mac239}-infected, NAb-negative rhesus macaques, respectively. SIV_{mac239}-binding capacity was screened by whole virus ELISA using virions purified from culture supernatants of SIV_{mac239}-infected HSC-F cells (a macaque T-cell line) (Figure 1). The measured absorbance was proportionate with Env gp120 and Gag p27 reactivity examined by immunoblotting (Figure 2). Polyclonal IgG lots from three macaques (R06-007, R01-009, and R03-005) with intermediate to high virion-binding capacity, although what percentage of IgGs was SIV-specific are unknown, were pooled and further used as a non-NAb cocktail for passive immunization, whose virion-binding characteristics were also confirmed (Figure 1).

To examine the *in vitro* virus-suppressive activity of the non-NAb cocktail, ADCVI activity was evaluated using PBMCs as effectors and MHC-mismatched macaque HSC-F cells as infected targets (Figure 3). IgG lots with high virion-binding capacity showed high ADCVI activity, whereas those from macaques R04-011 and R06-005 with limited reactivity in

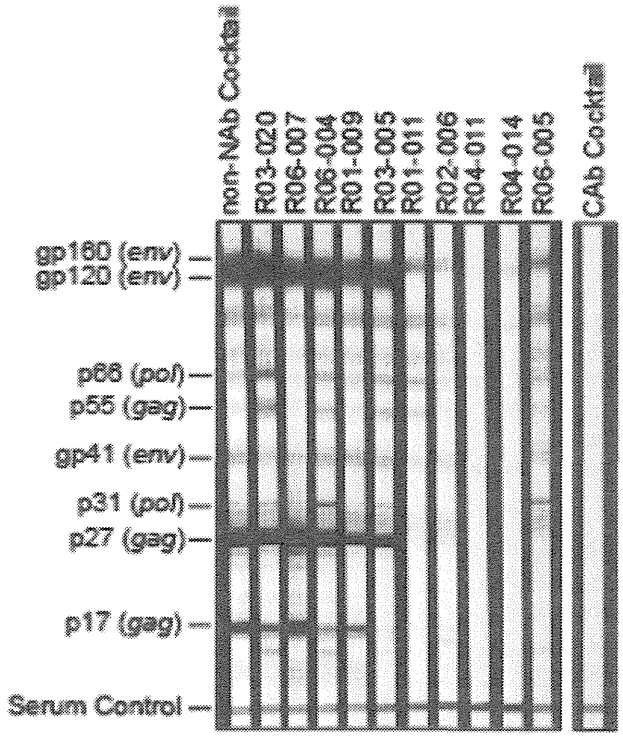

Figure 2. Binding properties of IgGs to SIV antigens. The non-NAb cocktail, ten non-NAb IgG lots derived from ten macaques, and CAb were subjected to immunoblotting (ZeptoMetrix). A representative result from two experiments is shown.

doi: 10.1371/journal.pone.0073453.g002

ELISA and western blot exhibited low ADCVI activity. These results suggest that ADCVI activity is proportionate with overall virion binding. The non-NAb cocktail exerted more than 97% inhibitory activity even at 0.1 mg/ml IgG concentration. A 1.0 mg/ml IgG concentration approximates an estimated *in vivo* antibody concentration immediately after passive immunization (300 mg IgG in 300 ml body fluid), implying that the observed ADCVI activity is likely to occur *in vivo* after passive immunization.

In Vivo Effect of Non-NAb Passive Immunization in SIV Infection

Having confirmed the *in vitro* anti-viral property of the non-NAb cocktail, we performed the post-infection passive immunization. Five rhesus macaques were challenged intravenously with SIV_{mac239} followed by passive immunization with the non-NAb cocktail (300 mg IgG) at day 7 post-challenge. When we previously passively immunized rhesus macaques with polyclonal antibodies having anti-SIV neutralizing activity by this regimen (300 mg IgG i.v. at day 7), enhanced virus uptake by DCs, subsequent augmentation of SIV-specific $CD4^+$ T-cell responses, enhancement of *in vitro* virus-suppressive activity in $CD8^+$ cells, and set-point viremia control were observed [20,21]. The current passive immunization experiment contrasts this previous report by

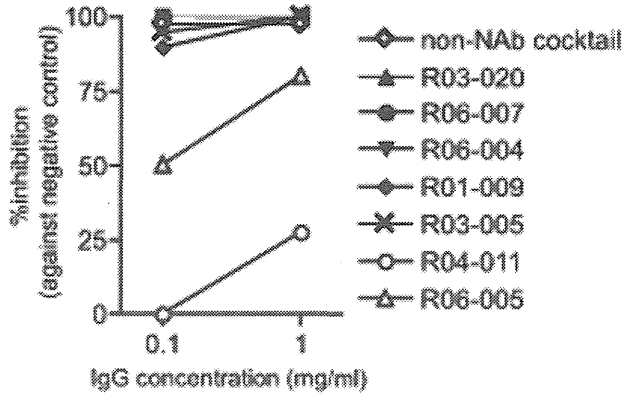

Figure 3. ADCVI activity of the non-NAb cocktail and non-NAb IgG lots. The reduction in SIV p27 concentration in the supernatant from SIV-infected cell culture with non-NAbs compared to that without antibodies is shown. A representative result, means of duplicate samples, from two experiments is shown.

doi: 10.1371/journal.pone.0073453.g003

infusion of polyclonal antibodies with comparable SIV virion-binding capacity and ADCVI activity without anti-SIV neutralizing activity. The moment of passive immunization (day 7) also recapitulates the first time frame to detect anti-HIV/SIV antibodies after infection [30,31]. Six animals consisting of two without passive immunization and four with control IgG infusion at day 7 after SIV_{mac239} challenge were used as controls.

To examine the abundance of non-NAbs after infusion and *de novo* virus-specific antibody induction, plasma reactivity against SIV antigens was measured by immunoblotting (Table 1). SIV Env-specific antibodies were detected at week 1.5 post-infection exclusively in the non-NAb-infused animals. High reactivity in plasma in these animals resided up to week 3 post-infection. *De novo* induction of SIV-specific antibodies was comparably observed in both the non-NAb-infused and control groups from week 5 to week 12 post-infection. Collectively, the passive non-NAb immunization resulted in systemic distribution of SIV Env-specific antibodies around peak infection.

All five macaques infused with the non-NAbs failed to contain set-point viremia, similar to the six control animals (Figure 4). The non-NAb-infused and control groups exhibited comparable peak and set-point viral loads without significant difference. No significant difference in total $CD4^+$ T-cell counts was found throughout the course between these two groups (Figure 4). Peripheral $CD95^+CD28^+$ central memory $CD4^+$ T-cell counts at week 12 were also comparable between these two groups (data not shown).

Considering our previous study of NAb-triggered SIV control and facilitation of T-cell responses [20,21], we examined SIV antigen-specific $CD8^+$ T-cell responses in the chronic phase (Figure 5). Neither the responses to individual antigens nor the summation presented significant difference. Finally, to assess possible selective pressure on SIV by the passive non-NAb immunization, predominant nonsynonymous env mutations in the early phase (at week 12, data not shown) and in the

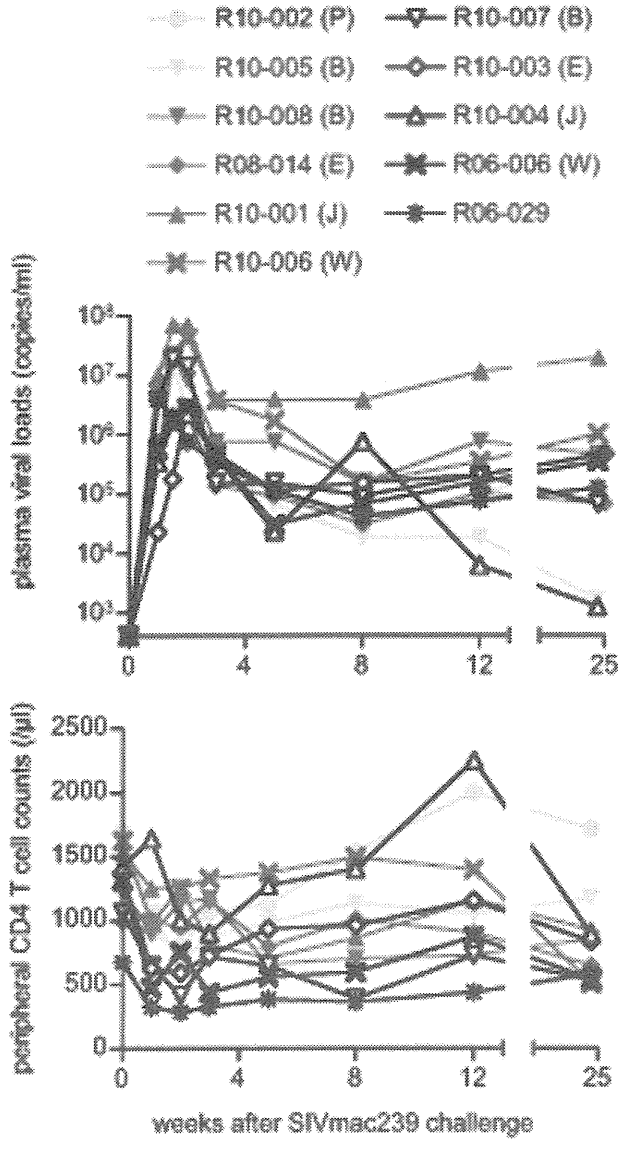

Figure 4. Passive non-NAb immunization in SIV infection. Upper panel: plasma viral loads after SIV_{mac239} challenge (SIV RNA copies/ml in plasma) in two unimmunized (green lines), four control IgG-immunized (blue), and five non-NAb-immunized macaques (black). Viral loads were determined as described previously [25]. The lower limit of detection is approximately 4 x 10^2 copies/ml. MHC-I haplotypes determined in individual animals are shown in parentheses as follows: B, haplotype *90-120-Ib*; E, *90-010-Ie*; J, *90-088-Ij*; P, *89-002-Ip*; W, *89-075-Iw*. Lower panel: peripheral $CD4^+$ T-cell counts after SIV_{mac239} challenge. No significant difference in viral loads or $CD4^+$ T-cell counts was observed between non-NAb-immunized and control animals.

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chronic phase were determined (Figure 6). Analysis at week 12 showed only one or two mutations, which were mostly observed also in the chronic phase. Mutations specific for the

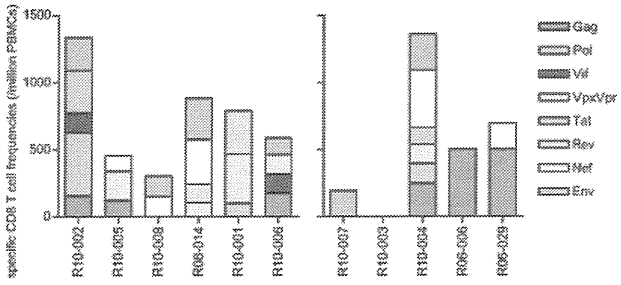

Figure 5. SIV antigen-specific $CD8^+$ T-cell responses. SIV Gag-, Pol-, Vif-, Vpx/Vpr-, Tat-, Rev-, Nef-, and Env-specific $CD8^+$ T-cell responses were measured by detection of antigen-specific IFN-γ induction using PBMCs at weeks 26-30 post-challenge.

doi: 10.1371/journal.pone.0073453.g005

Table 1. SIV-specific antibody responses in plasma after SIV infection.

macaques	regimens[a]	plasma antibody responses[b]				
		wk 1	wk 1.5	wk 3	wk 5	wk 12
R10-002	-	-	-	-	+	++++
R10-005	-	-	-	-	+	++++
R10-008	CAb	-	-	-	++	++++
R08-014	CAb	-	-	+	++	++++
R10-001	CAb	-	-	-	+	+
R10-006	CAb	-	-	-	+	++++
R10-007	non-NAb	-	+++	++	++	++++
R10-003	non-NAb	-	++++	++	++	++++
R10-004	non-NAb	-	+++	++	+	++++
R06-006	non-NAb	-	++++	++	+	++++
R06-029	non-NAb	-	+++	++	++	++++

[a] Animals received no passive immunization (-), passive CAb immunization (CAb), or passive non-NAb immunization (non-NAb) at day 7 after SIV challenge. [b] Antibody responses were detected using a commercial Western blotting system (ZeptoMetrix). + Gag p27-positive; ++ Gag p27 and Env gp160-positive; +++ Gag p27, Env gp160, and one other Gag/Pol/Env-derived antigen-positive; ++++ Gag p27, Env gp160, and two or more other Gag/Pol/Env-derived antigen-positive.

doi: 10.1371/journal.pone.0073453.t001

non-NAb-infused group, such as signs of ADCVI-induced escape [32], were not detected. A slight increase in predominant mutations in the Env V1-coding region was observed in the non-NAb-infused group, although the difference was not statistically significant (p = 0.08 by Mann–Whitney U test). Thus, the passive non-NAb immunization at day 7 post-challenge showed no significant impact on SIV replication *in vivo*.

Discussion

Whether augmentation of ADCVI without virus neutralizing activity may influence SIV replication control *in vivo* was a major interest in this study. Our results indicate that passive

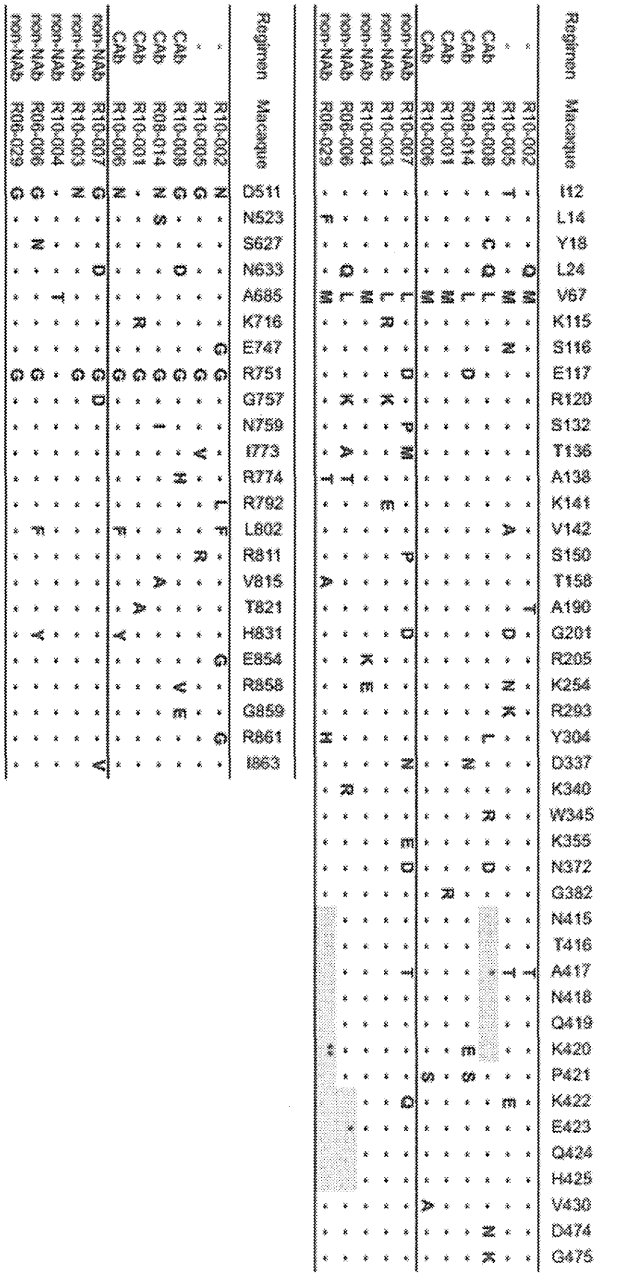

Figure 6. Predominant nonsynonymous env mutations. Viral cDNAs encoding Env were amplified from plasma RNAs obtained at 7-9 months (R10-001, R10-004, and R06-029) or 12 months (other animals) and subjected to sequencing analysis. Amino acid substitutions are shown. The asterisk (*) represents a deletion and the double asterisk (**) represents coexistence of multiple deletion patterns.

doi: 10.1371/journal.pone.0073453.g006

non-NAb immunization does not influence primary SIV replication when administered at early post-infection. In agreement with the limited protective effect observed when non-NAbs were administered locally on mucosa before virus challenge [16], systemic distribution of non-NAbs post-infection did not correlate with suppression of SIV replication, despite antiviral activity of the non-NAbs observed *in vitro*.

For assessment of antibody binding affinity to antigens on virions, we utilized purified SIV virions instead of recombinant Env proteins [33] as the antigen for ELISA under detergent-free conditions. Virion-binding characteristics of antibodies showed a similar trend with ADCVI activity, as seen in other studies [13]. However, the non-NAb infusion did not result in SIV control *in vivo*, which consequently proposes the following notions.

First, this study indicates that augmentation of non-NAb-derived virus-suppressive activity does not alter SIV control course once infection is achieved. While previous studies on immunized macaques indicated inverse correlation between ADCC or ADCVI activity at virus challenge and acute plasma viremia [11–14], the degree of non-NAb contribution by itself was not clear since vaccination elicited multiple immune responses. In other reports, intracutaneously infused non-NAbs did not exert protection in neonatal macaques [15] and mucosal non-NAbs showed limited protective activity [16]. In coherence, even massive systemic distribution of non-NAbs at peak infection did not impact viral replication in the present study. Thus, antiviral non-NAb responses do not suffice for counteracting establishment of set-point viremia, although these responses may partially influence viral replication in the chronic phase, as indicated by a previous report showing that CD20 depletion in chronic SIV infection can result in accelerated viremia and disease course [34], similar to rapid progressors [35]. Taken together, our results suggest that non-NAbs may withhold a limited role in impeding virus spread *in vivo* in HIV/SIV infections, unlike in other chronic viral infection models [36].

Second, this study indicates the requirement of neutralizing activity of antibody for the suppression of primary SIV replication by passive NAb immunization post-infection, as observed in our previous study [20]. The NAb-triggered SIV control has been suggested to be attributed to antibody-mediated virion uptake by DCs and enhanced T-cell priming [21], which can also occur in the non-NAb-infused animals. Thus, the control failure in non-NAb-infused macaques implies that augmentation of antigen presentation alone may be insufficient for primary SIV control and that reduction of infectious virus burden and $CD4^+$ T-cell preservation is important for any immune augmentation [20,37,38].

In conclusion, the post-infection passive non-NAb immunization did not result in primary SIV control in a rhesus macaque AIDS model. Our results suggest that virion binding and ADCVI activity with lack of virus neutralizing activity in the acute phase are insufficient for giving an impact on primary HIV/SIV replication. Further sophistication of local and targeted induction of functional non-NAb responses may be required to impact HIV/SIV replication *in vivo*.

Acknowledgements

We thank F. Ono, K. Hanari, K. Komatsuzaki, A. Hiyaoka, K. Oto, H. Akari, and Y. Yasutomi for assistance in animal experiments.

Author Contributions

Conceived and designed the experiments: T. Nakane TM HY. Performed the experiments: T. Nakane T. Nomura SS MN. Analyzed the data: T. Nakane TM HY. Wrote the manuscript: T. Nakane TM HY. Performed MHC-I typing: TKN AK.

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Highly-Sensitive Allele-Specific PCR Testing Identifies a Greater Prevalence of Transmitted HIV Drug Resistance in Japan

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Abstract

Background: The transmission of drug-resistant HIV in newly identified infected populations has become an underlying epidemic which can be better assessed with sensitive resistance testing. Since minority drug resistant variants cannot be detected by bulk sequencing, methods with improved sensitivity are required. Thus, the goal of this study was to evaluate if transmitted drug resistance mutations at minority levels in Japanese patients could be identified using highly sensitive allele-specific PCR (AS-PCR).

Materials and Methods: Samples were taken from newly diagnosed HIV/AIDS cases at the National Nagoya Hospital from January 2008 to December 2009. All samples were bulk sequenced for HIV protease and reverse transcriptase. To detect minority populations with drug resistance, we used AS-PCR with mutation-specific primers designed for seven reverse transcriptase inhibitor resistance mutations, M41L, K65R, K70R, K103N, Y181C, M184V, and T215F/Y, and for three protease inhibitor resistance mutations, M46I/L and L90M.

Results: We studied 149 newly identified HIV cases. Bulk sequencing detected 8 cases with NRTI resistance mutations (one with A62V, one D67E, one T215D, one T215E, two with T215L and two T215S) and 15 with PI resistance mutations (one with N88D and 14 with M46I). Results obtained by AS-PCR and bulk sequencing demonstrated good concordance but the AS-PCR enabled the detection of seven additional drug-resistant cases (one M41L, two with K65R, two with K70R, and one M184V) in the RT region. Additionally, AS-PCR assays identified 15 additional cases with M46I, five with M46L and four cases with L90M in the protease region.

Conclusions: Using AS-PCR substantially increased the detection of transmitted drug resistance in this population from 15.4% to 26.8%, further supporting the benefit of sensitive testing among drug-naïve populations. Since the clinical impact of minority drug-resistant populations is not fully comprehended for all mutations, follow-up studies are needed to understand their significance for treatment.

Citation: Nishizawa M, Hattori J, Shiino T, Matano T, Heneine W, et al. (2013) Highly-Sensitive Allele-Specific PCR Testing Identifies a Greater Prevalence of Transmitted HIV Drug Resistance in Japan. PLoS ONE 8(12): e83150. doi:10.1371/journal.pone.0083150

Editor: Fabrizio Mammano, INSERM, France

Received August 9, 2013; **Accepted** October 30, 2013; **Published** December 16, 2013

Funding: This work was supported by a Grant-in-Aid for AIDS research from the Ministry of Health, Labour, and Welfare of Japan [H22-AIDS-004]. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

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Introduction

The use of combination antiretroviral therapy of (cART) has resulted in sustained reductions in morbidity and mortality from HIV infection [1,2]. Five classes of antiretrovirals (ARVs) are currently available in clinical use in Japan. However, selection of drug resistance mutations during cART is still a major issue affecting the clinical efficacy of ARVs and prognosis of HIV infected individuals [3,4].

A United States Department of Health and Human Services (DHHS) guideline recommends drug resistance testing for patients before they begin cART to guide their therapy[5]. Conventional bulk sequencing is used to detect drug resistance mutations in viral RNA from patient plasma, but the method generally does not detect mutants that comprise less than 20% of the viral population in individuals [4-7]. This detection limitation is a concern, both because transmitted minority variants might persist at low frequencies and most newly

diagnosed HIV infections are in persons who have been infected for several months to years, providing time for drug resistant viruses with reduced viral fitness to decay to levels that conventional testing is not able to detect [8-10]. Therefore, the ability to detect low-frequency variants below 20% would improve identification of infections involving drug-resistant viruses and better inform decisions on the selection of active ARVs, especially for persons initiating treatment with NNRTI regimens. To detect low-frequency variants, several methods were developed and used to analyze drug-naïve persons and drug-experienced persons [11-14]. Several studies have shown the advantages of highly sensitive drug resistance assays with women who received intrapartum single-dose nevirapine (SD-NVP) for the prevention of mother-to-child HIV transmission. These reports on testing for NVP resistance have found that drug resistance emerges more frequently and persists for longer than previously demonstrated by bulk-sequencing. Persisting minority NVP-resistant viruses may result in poor virologic responses when subsequent regimens contain nevirapine-related drugs [15-19].. We previously reported that highly-sensitive drug resistance testing that is based on allele-specific real-time PCR can detect minority drug-resistant variants both in infections reported to be wildtype and infections involving other resistance mutations as determined by bulk sequencing. As with majority-level resistance, the amount of low-frequency resistance in new infections reflects both the prevalence of cART use in the region and behavior that is inconsistent with prevention practices for persons on therapy [20].

Recently, it has been reported that the prevalence of drug-resistant HIV transmission among newly diagnosed patients analyzed by bulk sequencing is increasing in Japan, rising from 5.9% in 2003 to 8.3% in 2008 [21]. As the study concluded, this observation was seen not only for recently infected persons, but also chronically infected but recently diagnosed cases, raising concern over the amount of resistance detection lost due to reversion. Therefore, by use of a highly sensitive method in the current study we attempted to examine for the possibility and prevalence of transmitted drug resistant mutations hidden as minority populations.

Materials and Methods

Ethics statement

Specimens were anonymous residual diagnostic material from subjects who provided written consent for HIV testing. The Ethical Committee for Biomedical Science of the National Institute of Infectious Diseases determined that this testing did not involve identifiable human subjects and has approved the study.

Samples

The 192 plasma samples were collected from HIV/AIDS cases for drug resistance analysis from January 2008 to December 2009 in National Nagoya Hospital (Table 1). Among these, 149 cases of newly diagnosed HIV-1 subtype B-infected ART-naïve individuals were selected and analyzed in this study (Figure 1). All samples were collected as part of HIV surveillance studies under Institutional Review Board of National Institute of Infectious Diseases, and written informed consent was obtained from each patient. These samples were directly sequenced for HIV protease (PR) positions 1-99 and reverse transcriptase (RT) positions 1-240. Drug resistance mutations were defined according to the mutation list proposed by Bennett et al. 2009[22]. All testing was performed by the NIID AIDS Research Center in Tokyo, Japan[21].

Table 1. Demographics of samples.

		2008	2009	Total
Total		75	74	149
Gender	male	74	73	147
	female	1	1	2
	unknown	0	0	
Age	median(Q1, Q3)	39	38	39
Risk behavior	MSM	52	49	101
	Sexual	9	11	20
	MSM/Sexual	8	13	21
	Hemophiliac	1	0	1
	Unknown	5	1	6
VL	Median	9.70E+04	7.00E+04	7.90.E+04
	mode	1.10E+05	2.70E+04	1.10.E+05
CD4	average	199.7	225.4	212.0

doi: 10.1371/journal.pone.0083150.t001

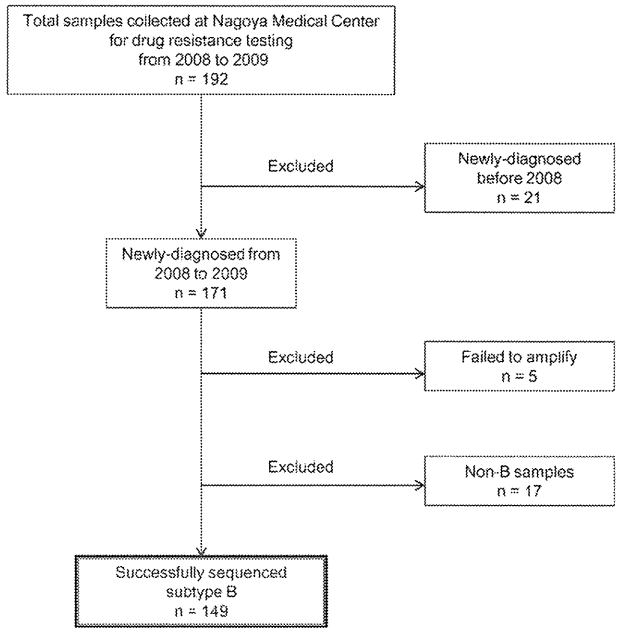

Figure 1. Flow Diagram of sample selection. Flow diagram of sample selection for analysis of minority drug resistance mutations in the HIV-infected patient samples newly diagnosed at National Nagoya Hospital from 2008 to 2009.

doi: 10.1371/journal.pone.0083150.g001

RNA extraction and virus template amplification

HIV RNA was extracted by Roche High Pure Viral RNA Kit from 200uL plasma samples. RNA was reconstituted in 100 uL of DEPC water and stored at -80°C until use. The HIV protease-reverse transcriptase (PR-RT) region was amplified by one-step RT-PCR (TAKARA One Step RNA PCR kit) with forward primer (DRPRO5 : AGA CAG GYT AAT TTT TTA GGG A) and reverse primer (DRRT34 : GCT ATT AAG TCT TTT GAT GGG TCA TA). RT-PCR amplification conditions were 55°C for 40 minutes and 40 cycles of 95°C for 10 seconds, 52°C for 5 seconds, and 72°C for 90 seconds. In the case that the amplification of RT- PCR did not generate sufficient template, nested-PCR was performed using forward primer (PROFWD1F : CAG ATC ACT CTT TGG CAA CGA CC) and reverse primer (GEN4R : ATC CCT GGG TAA ATC TGA CTT GC)[23]. Nested-PCR amplification condition was 94°C 1 minute and 30 Cycles of 94°C for 10 seconds, 55°C for 4 seconds and 74°C for 15 seconds.

Real-time PCR (AS-PCR)

To detect minority populations with drug resistance, we used highly sensitive allele-specific PCR validated for subtype B HIV as described [17,23]. Briefly, mutation-specific primers were designed for seven reverse transcriptase inhibitor resistance mutations, M41L, K65R, K70R, K103N, Y181C, M184V, and T215F/Y. Results of highly sensitive allele-specific PCR and population sequencing data were compared for concordance and presence of additional mutations. The HIV-1 total copy primers, Com2F and Com4BR, span n.t. 258–420 in RT and were used with the common probes, Com1P and 2P (Table S1) [17,23]. For multiple mutation screening, several resistance mutation-specific reactions can be performed simultaneously. The cycle number at which the fluorescence emission exceeds the background fluorescence threshold is the threshold cycle (CT) and is the unit of measure for comparing the differences in amplification signals (ΔCT) between the total copy and mutation-specific reactions. All samples were tested in duplicate with the means of the total copy and mutation-specific CTs used for the determination of the ΔCT. Each ΔCT cutoff value for interpreting the presence of drug resistance mutations was determined previously [23] and were between 8.5 from 10.5 cycles, for validated assay cut-offs ranging from 0.03% to 2.0% mutant, depending on the assay.

Real-time PCRs were initiated with a hot-start incubation at 94°C for 11 minutes before proceeding to 45 cycles of melting at 94°C for 30 seconds, annealing at 50°C for 15 seconds and extension at 60°C for 30 seconds. All reactions were performed in a total volume of 50 uL/well in 96-well PCR plates using iQ5 real-time PCR thermocyclers with optical units (Bio-Rad) and AmpliTaq Gold polymerase (2.5 U/reaction; Applied Biosystems). Final reagent concentrations were 320 nM for the forward and reverse primers, 160 nM probe(s), and 400 mM dNTPs.

M46I/L primers for real-time-PCR

For this study, new primers for the detection of M46I and M46L protease inhibitor mutations were constructed as described before [17,23,24]. As with the RT primers, the protease mutation-specific primers (Table S2) were designed to preferentially anneal with the targeted mutation nucleotide(s), thus having reduced affinity for wild-type sequences. Specificity was enhanced by creating designed mismatches at the -2 nucleotide position relative to the primer 3'-end for each primer. Furthermore, to compensate for the spectrum of polymorphisms present, mixtures of three uniquely designed forward primers were required to detect M46L. Mutation-specific primer mixtures were experimentally evaluated and the ratios that best balanced differences in primer avidities and minimized cross-interference in primer annealing were selected.

Site-directed mutagenesis and cloning

M46I and M46L mutant clones for plasmid development were constructed by site-directed mutagenesis using HXB2 as a template. These constructs were used as positive control to verify the M46I/L primers and probes. To insert M46I or M46L mutations into HXB2, PCR with KODplus was performed using a pair of complementary primers (M46I-forward primer : GAA GAT GGA AAC CAA AAA TaA TAG GGG GAA TTc GAG G, M46L(ttg)-forward primer : GAA GAT GGA AAC CAA AAt TGA TAG GGG GAA TTG GAG G , M46L(ctg)-forward primer : GAA GAT GGA AAC CAA AAc TGA TAG GGG GAA TTG GAG G), and reverse primer : CTG GCA AAC TCA TTT CTT CTA ATA CTG TAT CAT CTG CTC C). PCR amplification conditions were 94°C for 2 minutes and 35 cycles of 98°C for 10 seconds, 68°C for 2 minutes and 30 seconds.

Evaluation of the new protease assays on plasmids and clinical samples

HXB2-M46I, HXB2-M46L and HXB2 (wild-type) plasmids were used in the preliminary selection of primer mixtures that provided the greatest sensitivity and specificity. The absolute mutation detection limits for the primer mixtures, that is, the greatest ΔCT that was able to distinguish mutant viruses from wild-type, were estimated from triplicate testing of mutant clone serial dilutions. The assays evaluated mutation-containing sequences at frequencies between 100%-0.0001% in a wild-type background, with each dilution having same total plasmid copies. The ΔCTs generated from the mutant dilutions were compared to the ΔCTs generated with the wild-type plasmids alone. Solely for the purpose of comparing relative assay detection limits with finite virus sequences, the ΔCT within the linear dilution range (R^2>0.995) that was equivalent to a frequency increase of 0.5 $\log_{10}$ above the wild-type mean ΔCT was chosen as the absolute assay detection limit. Selecting the detection limit in this manner provided an adequate buffer against variability in wild-type sequences and also took into account the PCR efficiency of the assay (slope of the dilution curve).

To evaluate cutoff values of M46I and M46L in patient samples, PR-RT sequences derived from ART-naïve patients were analyzed by real-time PCR. Forty-two PR-RT region sequences derived from 16 patients were cloned by TA-cloning to serve as heterogeneous wild-type sequences. Fifty-five samples with protease M46I were obtained from 20 individuals and 22 samples with M46L were obtained from 12 individuals.

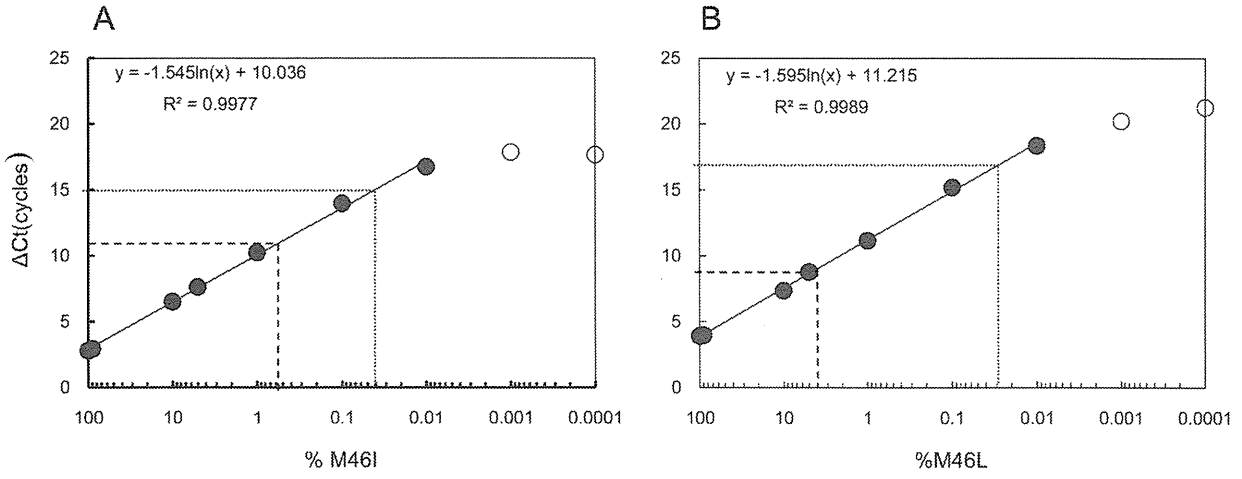

Figure 2. Mutation-specific assay reactivity on plasmids. Cloned M46I (A.) and M46L (B.) mutant virus template was diluted 10-fold, from 100% to 0.0001%, in backgrounds of wild-type sequence to determine assay detection limits. Plotted are the mean ΔCT versus $\log_{10}$ of the mutant dilution series. The lower detection limit (lower dotted line) was placed at the ΔCT equivalent to 0.5 $\log_{10}$ below (0.5-log greater reactivity than) the wild-type ΔCT. For comparison, the mutant virus frequency equivalences for the established clinical cutoffs are also shown (dashed line).

doi: 10.1371/journal.pone.0083150.g002

To increase the stringency of assay evaluations, specimens with substantial numbers of polymorphisms in primer binding sites were also included.

Assessing mutation associations in mutation-specific amplicons

To evaluate whether additional information on resistance mutations could be gained from the real-time PCR assays, we performed bulk sequencing (BigDye reagent, Prism 3130xl analyzer, Applied Biosystems) of the products from M46I/L or L90M-specific reactions to assess mutation linkage. Mutation-specific amplicon sequences were compared to their respective sample bulk sequence for evidence of nucleotide differences. Any other resistance mutation(s) found in the mutation-specific amplicons would indicate that they were on the same viral strand(s) as the mutation that was specifically targeted by the primers.

Phylogenetic analysis

Protease sequences were aligned by means of the clustal-W program with a set of reference sequences recommended by the Los Alamos sequence database (http://www.hiv.lanl.gov/content/index). The results of the alignment were then analyzed by the neighbor-joining method using MEGA5 [24,25]. In order to analyze the relationship between M46I/L-positive amplicon sequences and bulk sequences, we extended the M46I/L-positive amplicon by using the PRO2L reverse primer (Table S1B), which allowed sequencing from PR codon 47 to RT codon 36 of these M46I/L-positive amplicons (270 bp). In the case of L90M amplicon analysis, 209 bp DNA fragments extending from amino acid 20 in PR to amino acid 89 in PR were represented in the phylogenetic tree. The phylogenetic relatedness of these mutation-containing amplicons excluding the resistance codon position were represented in trees constructed using Kimura 2-parameter model with a discrete gamma distribution [1 +G] and 500 bootstrap replications in MEGA5.

Statistics

The Mann-Whitney U test was used to test for differences in CD4 counts and VL between the groups with minority drug resistance mutations and those without minority drug resistance mutations.

Results

Minority M46I could be detected as low as 0.04% and M46L could be detected as low as 0.03% in site-directed mutant clones

Relative limits of detection were compared in a simple laboratory setting using serial dilutions of HXB2-M46I or HXB2-M46L in backgrounds of HXB2 wildtype plasmid. The ΔCT that was equivalent to a 0.5 log greater reactivity than the wild-type mean ΔCT on the dilution curve (M46I : ΔCT=15 cycles, M46L : ΔCT=17 cycles) was used to compare assay sensitivities (Figure 2). This approach yielded detection limits of 0.04% and 0.03% for M46I and M46L, respectively. As this was derived from cloned sequences this is a theoretical detection limit against which clinical specimens are evaluated.

High sensitivity and specificity of M46I/L detection assays confirmed with clinical samples

Assay cutoff values intended for population-wide clinical screening were established using 42 cloned wild-type sequences derived from 14 patient-derived specimens

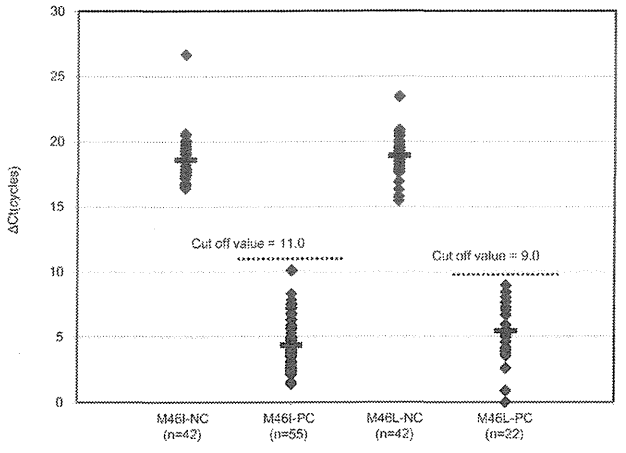

Figure 3. Assay reactivity with wild-type and M46I/L mutation clinical samples. Dotted ΔCT values from clinical samples with sequence-detectable mutations and with wild-type sequences are shown. The range of reactivity for each assay is shown for wild-type and mutant samples. The mean of ΔCT (bar) for each group is indicated. Assay cutoffs (dotted horizontal line) were established to exclude all wild-type viruses from detection. PC; Positive clones with M46I/L, NC; Negative clone with no M46I/L(wild type).

doi: 10.1371/journal.pone.0083150.g003

collected by NIID from 2005-2007. The assay cutoffs selected based on plasmid sequences were evaluated against clinical specimens using a total of 55 samples with sequence-detectable M46I mutation and a total 22 samples with sequence-detectable M46L mutation. The resulting distribution of collated ΔCTs from the wild-type samples supported a ΔCT cutoff of 11 cycles for M46I clinical testing (ΔCTs ranged from 16.39–26.65 cycles) (Figure 3 and Table S3). Extrapolating from the dilution curve for cloned M46I sequences, this cycle difference corresponded to a frequency mean of 0.54% mutant virus (see Table S3). At this cutoff, all 55 genotyped M46I samples were positive (ΔCTs ranged from 1.39 to 10.1 cycles, Figure 3 and Table S3). For M46L assay, ΔCT cutoff was 9 cycles to avoid low-level amplification from spurious primer binding against clinical quasispecies specimens; this cutoff placement corresponded to a frequency mean of 4.01% mutant virus. All genotype M46L samples were positive (ΔCT ranged from 0.88 to 8.95 cycles) (Figure 3 and Table S3). Because of unusual polymorphisms, some samples comprised almost entirely of mutant virus produced ΔCTs near the cutoff. In these situations, elevated ΔCTs resulting from weak primer binding could be interpreted as mutant viruses present at low frequencies. Hence, this testing format is best-suited to provide highly specific population-level resistance screening and is not necessarily applicable to mutant virus quantitation.

AS-PCR method identifies a greater prevalence of transmitted HIV drug resistance

Samples from a total of 149 subtype B cases were collected at Nagoya Medical Center for drug resistance testing. Drug resistance mutations were initially analyzed by bulk sequencing, and 23 cases were found to possess drug resistance mutations. As summarized in Table 2, all resistant mutations were found as sole mutation, one with A62V, one with D67E and six cases of intermediates at codon 215 (one with T215D, one with T215E, two cases of T215L and two cases of T215S), one with N88D and 14 cases of M46I mutation were detected by conventional bulk sequencing analysis, yielding a drug resistance mutation prevalence of 15.4% (23/149 cases) (Table 2). The sensitive screening detected an additional one case of M41L (0.67%), two cases of K65R (1.34%), two cases of K70R (1.34%), one case of M184V (0.67%), 15 cases of M46I (19.46%), 5 cases of M46L (3.36%) and 4 cases of L90M (2.68%) as minority-level drug resistance mutations (Table 2). The identified A62V, D67E, T215E, T215S and N88D mutations detected by bulk sequencing were not targeted by AS-PCR, and therefore were not included in determining changes in mutation frequency. All 17 mutations detected by bulk sequencing analysis that were also targeted by AS-PCR were likewise detected by the sensitive PCR method. The combined prevalence of drug resistance mutations in the total of 149 cases was 26.8% (40/149 cases) (Table 2). In one case, six mutations, M41L, K70R, M184V, M46I, M46L and L90M were detected as minority mutations by the highly sensitive assays (ID 29). These six mutations were undetectable by bulk sequencing analysis. In other cases, K70R and M46I were detected (ID 22), and M46I and M46L were detected in another case as minority drug resistance mutations (ID 5). Of those with minority drug resistance mutations, 11 cases were from 2008 and 12 cases were from 2009. The majority of patients with minority variants were MSM (90.9% in 2008 and 83.3% in 2009) and Japanese, and no significant differences were observed in viral load and CD4 counts by Mann-Whitney U test (p=0.17 and p=0.308, respectively) for persons with or without mutations. Though all of the samples from 2008 were Japanese patients, three cases from 2009 were non-Japanese patients (Table 2).

Sequence analysis of M46I and M46L-specific amplicons showed that these mutations were not linked to L90M in the patient samples

To analyze the linkage between drug resistance mutations, we directly sequenced positive M46I/L or L90M-specific PCR products to ascertain whether additional genotypic information could be obtained from those amplicons. In ID 29, the I72V polymorphism observed in the bulk sequence was detected in M46L and L90M amplicons, but not in the M46I amplicon (Table 3). Additionally, M46I/L mutations were not detected in the minority L90M amplicon indicating these mutations were not linked. In ID 27, A71T was detected in the M46L amplicon, but this mutation was not found in the M46I amplicon or the bulk sequence (Table 3). In ID 22, the M46I amplicon matched the bulk sequence.

Table 2. Characteristics of HIV/AIDS patients with drug resistance mutations.

							Bulk-seq		AS-PCR	
ID	Gender	Risk behavior	Year	Nationality	VL	CD4	RT mutations	PR mutations	RT mutations	PR mutations
1	M	MSM	2008	Japan	2.0.E+04	402	A62A/V			
2	M	Heterosexual	2008	Japan	1.2.E+06	14		N88D/N		
3	M	Heterosexual	2008	Japan	3.1.E+05	38	T215L		T215F*	
4	M	MSM/Heterosexual	2008	Japan	5.8.E+05	222	D67D/E			M46I
5	M	MSM	2008	Japan	2.6.E+04	481				M46I, M46L
6	M	MSM	2008	Japan	1.7.E+06	10				M46I
7	M	MSM/Heterosexual	2008	Japan	4.1.E+05	39	T215S			
8	M	MSM	2008	Japan	2.2.E+05	14		M46I		M46I
9	M	MSM	2008	Japan	1.5.E+04	356				L90M
10	M	MSM/Heterosexual	2008	Japan	1.2.E+05	28	T215S			
11	M	MSM/Heterosexual	2008	Japan	1.3.E+05	348		M46I		M46I
12	M	MSM	2008	Japan	2.1.E+04	391			K65R	
13	M	MSM	2008	Japan	2.1.E+05	553		M46I		M46I, L90M
14	M	Heterosexual	2008	Japan	6.7.E+04	153		M46I		M46I
15	M	MSM	2008	Japan	2.2.E+05	10		M46I		M46I, M46L
16	M	MSM	2008	Japan	1.2.E+03	45	T215L		T215F*	M46I
17	M	MSM	2008	Japan	2.6.E+04	750		M46I		M46I
18	M	MSM	2008	Japan	1.1.E+05	146		M46I		M46I
19	M	MSM	2008	Japan	8.4.E+04	11				M46I
20	M	MSM	2008	Japan	1.4.E+05	86		M46I		M46I
21	M	MSM	2008	Japan	1.1.E+05	1050				M46I
22	M	MSM	2008	Japan	2.2.E+04	154			K70R	M46I
23	M	MSM	2009	Japan	7.2.E+03	319				M46L
24	M	MSM	2009	Japan	2.9.E+04	185				M46I
25	M	Heterosexual	2009	Japan	1.6.E+04	290	T215D		T215Y**	
26	M	MSM/Heterosexual	2009	Japan	2.7.E+04	442		M46I		M46I
27	M	MSM	2009	Brazil	8.6.E+04	14		M46I		M46I, M46L
28	M	MSM	2009	Argentina	2.0.E+05	28				M46I
29	M	MSM	2009	Japan	4.5.E+05	3			M41L, K70R, M184V	M46I, LM46L, L90M
30	M	MSM	2009	Japan	1.7.E+05	nt		M46I		M46I
31	M	MSM	2009	Japan	2.9.E+05	32	T215E		T215Y	
32	M	MSM	2009	Japan	1.1.E+05	833		M46I		M46I
33	M	MSM	2009	Japan	4.1.E+04	426		M46I		M46I
34	M	MSM	2009	Japan	1.2.E+07	441		M46I		M46I
35	M	MSM	2009	Australia	1.5.E+04	1				M46I
36	M	MSM	2009	Japan	2.2.E+04	324				L90M
37	M	MSM	2009	Japan	6.0.E+04	373				M46I
38	M	MSM	2009	Japan	2.9.E+04	nt			K65R	
39	M	Heterosexual	2009	Japan	4.5.E+03	974				M46I
40	F	Heterosexual	2009	Japan	8.0.E+04	nt				M46I
41	M	MSM	2009	Japan	1.4.E+04	300				M46I
Subtotal							8(5.3%)	15(10.1%)	8(5.3%)	32(21.5%)
Total							23/149(15.4%)		40/149(26.8%)	

* T215F detection primers can detect T215L, T215I and T215V. **T215Y detection primers can detect T215D, T215H and T215N.

doi: 10.1371/journal.pone.0083150.t002

Phylogenetic relatedness of minority and bulk sequence-detectable resistance mutations

Phylogenetic analysis conducted on all the protease sequences produced a pattern consistent with good separation of unrelated sequences even though they were not supported at the roots by significant bootstrap values due to somewhat short sequence lengths (Figure 4A). However, the branch tips show strong bootstrap support for the relatedness of minority variants to the patient bulk sequences from which they were derived. Moreover, some of the patients with bulk sequence-detectable M46I appeared to group together with relatively high bootstrap values (pairs X and Y, Figure 4A) and may represent infections linked within transmission clusters. The sequences

Table 3. Genetic linkage of M46I/L or L90M and other mutations.

Sample	Sequences	Mutations
ID 29	Direct-sequencing	I62V, L63P, *I72V*, T74A, V77I, I93L
	M46I amplicon*	**M46I**, I62V, L63P, T74A, V77I
	M46L amplicon*	**M46L**, I62V, L63P, *I72V*, T74A, V77I
	L90M amplicon**	I62V, L63P, *I72V*, T74A, V77I, **L90M**
ID 27	Direct-sequencing	**M46I**, E21R, R41K, I62V, L63P, L89I, Q92K, I93L
	M46I amplicon*	**M46I**, I62V, L63P
	M46L amplicon*	**M46L**, I62V, L63P, *A71T*
ID 22	Direct-sequencing	E35D, M36I, L63P, H69K, V77I
	M46I amplicon*	**M46I**, L63P, H69K, V77I

* M46I and M46L amplicons were spanned from M46 to N88.

** L90M amplicon was spanned from I15 to L90.

doi: 10.1371/journal.pone.0083150.t003

within each X and Y pair were 100% identical in the 270 bp analyzed, with the exception of ≤3 mixed-base positions that included the nucleotide of the paired patient. In assessing the relatedness of the four detected minority L90M to infections that have the PR M46I mutation, three L90M were from patients that were wildtype at codon 46, the fourth was ID 13 which was also an M46I case (Figure 4B).

Discussion

In this study, we used a highly sensitive method to screen for minority drug-resistant populations in 149 cases of newly diagnosed HIV-infected patients. An additional six drug resistance mutations in the RT region and thirty drug resistance mutations in PR region were detected as minority-level drug resistance mutations. For the ten codons associated with resistance (RTI mutations: M41L, K65R, K70R, K103N, Y181C, M184V and T215F/Y, PI mutations: M46I/L and L90M) the prevalence of detectable drug resistance mutations increased from 15.4% to 26.8% using the highly sensitive assays. A previous surveillance study in Japan using bulk sequencing reported that in 2008 the prevalence of transmitted drug resistance was 8.3% [21]. Therefore, drug resistance mutation surveillance analyzed by bulk sequencing underestimates transmitted drug resistance, which potentially has both clinical and epidemiologic implications.

The epidemiologic implications of increased transmitted resistance may reflect prevention failures in persons who know they are infected and transmit HIV to their partners. The clinical implications of minority drug resistance center around the impact of these viruses on ART responses. Previous studies have reported that minority NNRTI-resistant variants are associated with increased risk of virologic failure in patients receiving first-line NNRTI-based ART regimens [20,26-32]. These findings are important because NNRTI resistance is the most commonly transmitted resistance in the US and Europe [33,34]. However, no evidence of either majority or minority NNRTI-resistance was found in this study, a unique finding that is explained by the historically infrequent use of NNRTIs in ART regimens in Japan. Instead, we note that the NRTI K65R

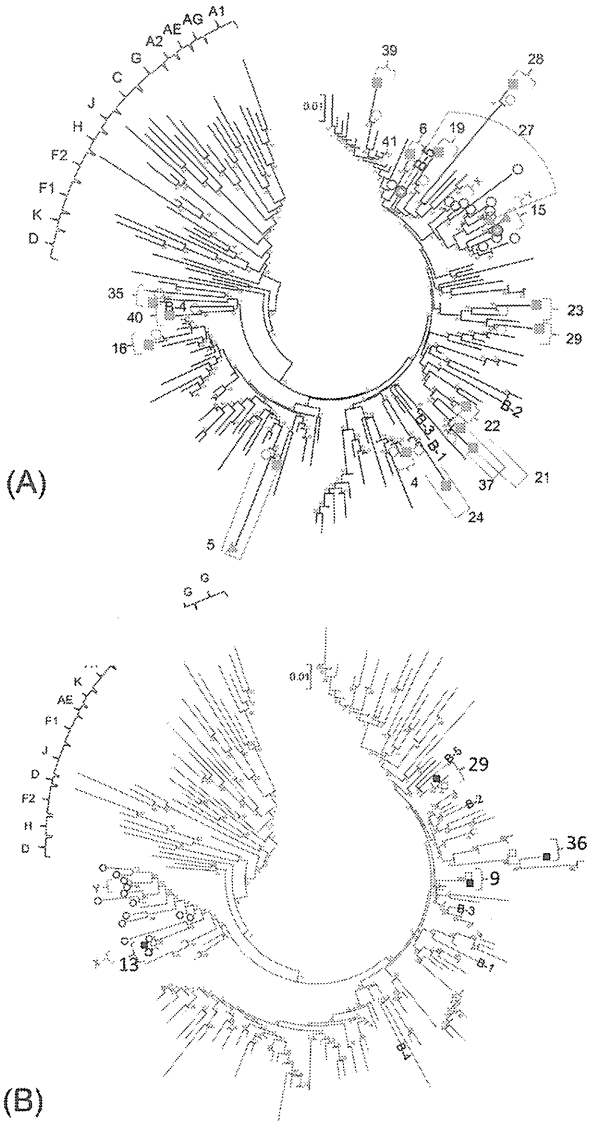

Figure 4. Phylogenetic tree of samples with or without minority variants of M46I/L or L90M drug resistance. A neighbor-joining phylogenetic tree of all protease sequences using the Kimura 2-parameter model was generated in MEGA5. Numbers shown are IDs of patients with detectable minority drug resistance. Open circles (black color) are virus with M46I detected by bulk sequencing. A. Solid squares (red color) indicate sequences of M46I-specific amplicons and solid triangle (red color) indicate sequences of M46L amplicons; open circles (red color) indicate bulk sequences for persons with minority M46I/L mutations; X and Y are pairs of closely related transmitted M46I sequences. B. Solid squares indicate L90M-specific amplicon sequences; open squares indicate bulk sequences for persons with minority L90M mutations. Open circles (black color) are virus with M46I detected by bulk sequencing. Abbreviations of subtype B references: B-1; B.NL.00.671 00T36.AY423387, B-2; B.US.98.1058 11.AY331295, B-3; B.FR.83.HXB2 LAI IIIB BRU.K03455, B-4; B.TH.90.BK132.AY173951, B-5; B.US.98.15384 1.DQ853463.

doi: 10.1371/journal.pone.0083150.g004

and M184V mutations were both detected as minority populations. As major mutations K65R and M184V reduce the clinical efficacy of TDF and 3TC/FTC, respectively [35-37], however their clinical impact as minority mutations is not fully clear.

A previous study demonstrated no impact on therapy responses in patients who had minority-level K65R and M184V mutations when provided regimens that included protease inhibitors; however; because of the small number of patients representing different treatment regimens in that study, the bearing of these mutations could not be fully evaluated [29]. Additional studies are needed to assess clinically significant frequencies of different NRTI-resistant variants on various treatment regimens. In the present study, one of the 149 cases evaluated possessed six drug resistance mutations as minority variants (Table 2). Genotype interpretation by the Stanford HIV Drug Resistance Database showed that the patient possessed high-level resistance to NRTIs and some PIs. It was not possible to follow the clinical course of this patient to elucidate the significance of minority variants on subsequent cART.A major finding in this study was the high prevalence of transmitted PI resistance (20%) which accounted for about two-thirds the overall transmitted resistance. The high prevalence of transmitted PI resistance is supported both by detection at majority as well as minority variant levels, the latter comprising more than half of the transmitted PI cases. The high prevalence of PI resistance can be explained by the longstanding and predominant use of PI-based regimens in Japan, including darunavir and atazanavir in both first-line and second-line regimens

Genetic linkage analysis provided more insights into the composition of the viral population by showing that L90M, M46I and M46L in many patients existed on separate viral genomes. The capacity to identify linked mutations could be important for understanding the persistence [38] and clinical impact of mutant variants. A major factor that influences the persistence of drug-resistant mutants *in vivo* is their relative replicative capacity within the viral population. *In vitro* competition experiments conducted in the absence of drugs have shown that drug resistance mutations impair replicative fitness by different degrees. For instance, the M46I, the K70R, the 215 intermediate mutations have a lesser impact on fitness than L90M, K65R, and M184V [38-40], and, thus, such mutations are likely to persist longer *in vivo*. Moreover, accumulation of compensatory mutations such as L63P and A71V in protease have been demonstrated to increase or restore replicative fitness of PI resistant variants, and that once compensation has taken place reversion to wildtype is prohibited by a less fit intermediate [41]. This may explain, for example, the high prevalence of M46I we detected in this study as bulk and minority species.

Phylogenetic analysis showed the sequences of minority M46I/L and L90M-positive amplicons were closely related to their source bulk sequences, supporting that the minority sequences detected were unique to the respective patients and were not the result of contamination. A few cases possessing the M46I mutation by bulk sequencing demonstrated a strongly supported identity which was not biased by including the resistance mutations in the analysis. However, with regard to minority M46I variants, we found they did not cluster closely with viruses from persons with majority-level M46I. This suggested that at least two pairs with majority-level M46I were phylogenetically related whereas those with minority M46I were scattered among the transmitted virus population, unrelated to any of the other cases in our analysis. While the sequence lengths used in the analysis might limit our ability to draw robust bootstrap values deeper in the tree nodes, the sequences for the pairs within the two clusters were identical with the exception of a few positions with mixed bases, and were further supported by high bootstraps.

The ability to conduct surveillance of minority-level drug resistance mutations is an important advancement to help understand transmission of HIV drug resistance in Japan. The finding from our select analysis of mutations of interest cannot be extrapolated to all codons associated with drug resistance; however, these results suggest that a substantial proportion of drug resistance-associated mutations are persist at low levels by the time HIV-infected persons are diagnosed and genotyped. Using an approach that can more broadly identify variants, such as next-generation sequencing [42-45], may identify other mutations that would further increase the prevalence of drug resistance. However, because the more commonly transmitted mutations are often targeted by AS-PCR, any additional increase in mutation prevalence identified by the more complex methods may be nominal. Hence, the lower cost and simplicity of AS-PCR offer advantages for routine surveillance, particularly when the sample burden may be high.

In conclusion, the relationship between minority drug resistance mutations and cART failure requires further exploration; nevertheless, the findings point to difficulties in getting infected persons diagnosed early and counseled to prevent forward spread of drug resistance.

Acknowledgements

Disclaimer: The findings and conclusions presented in this manuscript are those of the authors and do not necessarily represent the official views of the Centers for Disease Control and Prevention. We thank William Switzer for assistance with the phylogenetic analysis.

Author Contributions

Conceived and designed the experiments: WS JJ WH. Performed the experiments: MN JH. Analyzed the data: MN JH ST WS. Contributed reagents/materials/analysis tools: JJ WH WS TM. Wrote the manuscript: MN JJ WH WS.

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Control of Simian Immunodeficiency Virus Replication by Vaccine-Induced Gag- and Vif-Specific CD8$^+$ T Cells

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For development of an effective T cell-based AIDS vaccine, it is critical to define the antigens that elicit the most potent responses. Recent studies have suggested that Gag-specific and possibly Vif/Nef-specific CD8$^+$ T cells can be important in control of the AIDS virus. Here, we tested whether induction of these CD8$^+$ T cells by prophylactic vaccination can result in control of simian immunodeficiency virus (SIV) replication in Burmese rhesus macaques sharing the major histocompatibility complex class I (MHC-I) haplotype *90-010-Ie* associated with dominant Nef-specific CD8$^+$ T-cell responses. In the first group vaccinated with Gag-expressing vectors ($n = 5$ animals), three animals that showed efficient Gag-specific CD8$^+$ T-cell responses in the acute phase postchallenge controlled SIV replication. In the second group vaccinated with Vif- and Nef-expressing vectors ($n = 6$ animals), three animals that elicited Vif-specific CD8$^+$ T-cell responses in the acute phase showed SIV control, whereas the remaining three with Nef-specific but not Vif-specific CD8$^+$ T-cell responses failed to control SIV replication. Analysis of 18 animals, consisting of seven unvaccinated noncontrollers and the 11 vaccinees described above, revealed that the sum of Gag- and Vif-specific CD8$^+$ T-cell frequencies in the acute phase was inversely correlated with plasma viral loads in the chronic phase. Our results suggest that replication of the AIDS virus can be controlled by vaccine-induced subdominant Gag/Vif epitope-specific CD8$^+$ T cells, providing a rationale for the induction of Gag- and/or Vif-specific CD8$^+$ T-cell responses by prophylactic AIDS vaccines.

Human immunodeficiency virus (HIV) infection induces persistent viral replication, leading to AIDS onset in humans. Virus-specific CD8$^+$ T-cell responses play a central role in the resolution of acute peak viremia (1–4) but mostly fail to contain viral replication in HIV infection. Prophylactic vaccination resulting in more effective CD8$^+$ T-cell responses postexposure than those in natural HIV infections might contribute to HIV control. Current trials in macaque AIDS models have shown that vaccine induction of T-cell responses can result in control of postchallenge viral replication (5–10). It is now critical to define the antigens that elicit the most potent responses for development of an effective T-cell-based AIDS vaccine.

Recent studies have implicated Gag-specific CD8$^+$ T cells in the control of HIV and simian immunodeficiency virus (SIV) replication (11–16). Several HLA or major histocompatibility complex class I (MHC-I) alleles have been shown to be associated with lower viral loads (17–25). Virus control associated with some of these protective MHC-I alleles is attributed to Gag epitope-specific CD8$^+$ T-cell responses (26–29). For instance, CD8$^+$ T-cell responses specific for the HLA-B*57-restricted $Gag_{240–249}$ TW10 and HLA-B*27-restricted $Gag_{263–272}$ KK10 epitopes exert strong suppressive pressure on HIV replication and frequently select for escape mutations with viral fitness costs, leading to lower viral loads (27, 30–33). Thus, certain individuals possessing MHC-I alleles associated with dominant Gag-specific CD8$^+$ T-cell responses could have a greater chance to control HIV replication than those without these alleles. For those individuals that do not express these MHC-I alleles, the question arises as to whether prophylactic vaccination inducing Gag epitope-specific CD8$^+$ T-cell responses might contribute to HIV control. Furthermore, recent studies have shown that CD8$^+$ T-cell responses targeting SIV antigens other than Gag, such as Mamu-B*08- or Mamu-B*17-restricted Vif and Nef epitopes, exert strong suppressive pressure on SIV replication (10, 34, 35).

We previously developed a prophylactic AIDS vaccine consisting of a DNA prime and a boost with a Sendai virus (SeV) vector expressing SIVmac239 Gag (SeV-Gag) (36). Our trial showed vaccine-based control of an SIVmac239 challenge in a group of Burmese rhesus macaques sharing the MHC-I haplotype *90-120-Ia* (5, 37). Unvaccinated animals possessing *90-120-Ia* dominantly elicited CD8$^+$ T-cell responses specific for the $Gag_{206–216}$ (IINEE AADWDL) and the $Gag_{241–249}$ (SSVDEQIQW) epitopes after SIV challenge (38, 39). DNA/SeV-Gag-vaccinated *90-120-Ia*-positive macaques showed enhanced $Gag_{206–216}$-specific and $Gag_{241–249}$-specific CD8$^+$ T-cell responses in the acute phase after SIV challenge, resulting in viremia control (37). This implies virus control by vaccine-based enhancement of Gag-specific CD8$^+$ T-cell responses in animals possessing MHC-I alleles associated with dominant Gag CD8$^+$ T-cell epitopes. However, we have not defined the efficacy of prophylactic vaccination inducing Gag-specific CD8$^+$ T-cell responses against HIV/SIV infection in the hosts pos-

Received 11 September 2013 Accepted 17 October 2013
Published ahead of print 23 October 2013
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/JVI.02634-13.

doi:10.1128/JVI.02634-13

sessing MHC-I alleles not associated with dominant Gag $CD8^+$ T-cell epitopes.

In the present study, we first examined efficacy of prophylactic vaccination inducing Gag-specific $CD8^+$ T-cell responses against SIVmac239 challenge in a group of macaques that possess the *90-010-Ie* MHC-I haplotype (referred to as E) associated with dominant Nef-specific $CD8^+$ T-cell responses (39, 40). Furthermore, we examined the efficacy of prophylactic vaccination inducing Vif/Nef-specific $CD8^+$ T-cell responses in these E^+ macaques. Our results show SIV control in those vaccinees that mounted efficient Gag- or Vif-specific $CD8^+$ T-cell responses in the acute phase postchallenge.

MATERIALS AND METHODS

Animal experiments. Animal experiments were carried out in Tsukuba Primate Research Center, National Institute of Biomedical Innovation (NIBP), with the help of the Corporation for Production and Research of Laboratory Primates after approval by the Committee on the Ethics of Animal Experiments of NIBP (permission number DS21-28 and DS23-19) under the guideline for animal experiments at NIBP and National Institute of Infectious Diseases, which is in accordance with the Guidelines for Proper Conduct of Animal Experiments established by Science Council of Japan (http://www.scj.go.jp/ja/info/kohyo/pdf/kohyo-20-k16-2e.pdf). Blood collection, vaccination, and SIV challenge were performed under ketamine anesthesia.

We used Burmese rhesus macaques (*Macaca mulatta*) possessing the MHC-I haplotype *90-010-Ie* (E) (39, 40). The determination of 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 as described previously (39–41). Confirmed MHC-I alleles consisting of the MHC-I haplotype E are *Mamu-A1*066:01*, *Mamu-B*005:02*, and *Mamu-B*015:04*. Unvaccinated R01-011, R05-007, R08-003, R08-007, R09-011, and R06-038 and Gag-vaccinated R01-010 and R01-008 used in our previous experiments (39, 42) are included in the present study. At week 1, unvaccinated macaque R06-038 was intravenously infused with 300 mg of nonspecific immunoglobulin G purified from uninfected rhesus macaques as described before (43). All animals were intravenously challenged with 1,000 50% tissue culture infective doses ($TCID_{50}$) of SIVmac239 (44).

Macaques R01-010, R05-010, R01-008, R08-002, and R08-006 received prophylactic DNA prime/SeV-Gag boost vaccination (referred to as Gag vaccination) (5). The DNA used for the vaccination, cytomegalovirus (CMV)-SHIVdEN, was constructed from *env*-deleted and *nef*-deleted simian-human immunodeficiency virus SHIVMD14YE (45) molecular clone DNA (SIVGP1) and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx and HIV Tat and Rev. At the DNA vaccination, animals received 5 mg of CMV-SHIVdEN DNA intramuscularly. Six weeks after the DNA prime, animals received a single boost intranasally with 6×10^9 cell infectious units (CIU) of F-deleted replication-defective Sendai virus (SeV) expressing SIVmac239 Gag (SeV-Gag) (46).

Macaques R08-012, R10-012, R10-013, R10-010, R10-011, and R10-014 received prophylactic DNA prime/SeV-VifNef boost vaccination (referred to as Vif/Nef vaccination). The Vif-expressing DNA used for the vaccination, pcDNA-SIVvif-opt, was constructed by introducing an optimized SIVmac239 Vif cDNA (GenScript) into pcDNA3.1. The Nef-expressing DNA used for the vaccination, pcDNA-SIVnef-G2A, has an SIVmac239 Nef cDNA with a mutation resulting in glycine (G) to alanine (A) at the 2nd amino acid (aa) in Nef. Animals intramuscularly received 3 mg of Vif-expressing DNA at the first DNA vaccination and 3 mg of Vif-expressing DNA and 3 mg of Nef-expressing DNA at the second DNA vaccination. Six weeks after the first DNA prime, animals received a single boost intranasally with 1×10^9 CIU of F-deleted SeV expressing Vif-opt (SeV-Vif) and 1×10^9 CIU of F-deleted SeV expressing Nef-G2A (SeV-Nef) (47).

Analysis of antigen-specific $CD8^+$ T-cell responses. We measured virus-specific $CD8^+$ T-cell frequencies by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific stimulation as described previously (48, 49). Autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines (B-LCLs) were pulsed with each peptide (at a final concentration of 1 μM) or peptide pools (at a final concentration of 1 to 2 μM for each peptide) using panels of overlapping peptides spanning the entire SIVmac239 Gag, Vif, and Nef amino acid sequences (Sigma-Aldrich Japan) for 1 h. Peripheral blood mononuclear cells (PBMCs) were cocultured with these pulsed B-LCLs in the presence of GolgiStop (monensin; BD) for 6 h. Intracellular IFN-γ staining was performed with a Cytofix/Cytoperm kit (BD) and fluorescein isothiocyanate-conjugated anti-human CD4 (BD), peridinin chlorophyll protein-conjugated anti-human CD8 (BD), allophycocyanin (APC)-Cy7-conjugated anti-human CD3 (BD), and phycoerythrin (PE)-conjugated anti-human IFN-γ monoclonal antibodies (BioLegend). In the flow cytometric analysis, PBMCs were gated in forward scatter-side scatter dot plots, and B-LCLs were excluded in this step. Specific T-cell frequencies were calculated by subtracting nonspecific IFN-γ T-cell frequencies (less than 100 per million PBMCs) from those after peptide-specific stimulation. Specific T-cell frequencies lower than 100 per million PBMCs were considered negative.

Sequencing analysis of plasma viral genomes. Viral RNAs were extracted using the high pure viral RNA kit (Roche Diagnostics, Tokyo, Japan) from macaque plasma obtained around 1 year after challenge. Fragments of cDNAs encoding SIVmac239 Gag, Vif, and Nef were amplified by nested RT-PCR (25 cycles at the first RT-PCR using the PrimeScript one-step RT-PCR kit, version 2 [TaKaRa] and 30 cycles at the second PCR using KOD-Plus, version 2 [Toyobo]) from plasma RNAs and subjected to direct sequencing by using dye terminator chemistry and an automated DNA sequencer (Applied Biosystems, Tokyo, Japan) as described before (39). Predominant nonsynonymous mutations were determined.

Statistical analysis. Statistical analysis was performed with Prism software version 4.03, with significance levels set at a P value of <0.050 (GraphPad Software, Inc.). Antigen-specific $CD8^+$ T-cell frequencies were compared by the nonparametric Mann-Whitney U test. Correlation was analyzed by the Pearson test.

RESULTS

Plasma viral loads after SIVmac239 challenge. We used a group of Burmese rhesus macaques possessing the MHC-I haplotype *90-010-Ie* (E). In our previous study (39), unvaccinated E^+ macaques consistently showed persistent viremia after SIVmac239 challenge. $CD4^+$ T-cell percentage in PBMCs declined to less than 20% in a year. In the present study, we compared viral loads in vaccinated animals with those in these unvaccinated animals.

The first vaccine group of five E^+ macaques received a DNA prime and an SeV-Gag boost vaccination, followed by an SIVmac239 challenge. Two of these Gag-vaccinated animals failed to control viral replication, but the remaining three showed SIV control (Fig. 1). In the latter controllers, plasma viremia became undetectable in a few months. Macaques R01-008 and R08-006 rapidly controlled SIV replication and maintained high CD4 levels (Fig. 1).

The second group of six E^+ macaques received a DNA prime and an SeV-Vif/Nef boost vaccination, followed by an SIVmac239 challenge. The vaccine protocol first delivered Vif-expressing DNA, with the second vaccination consisting of Vif-expressing and Nef-expressing DNAs, and the third with Vif-expressing and Nef-expressing SeVs (SeV-Vif and SeV-Nef) with intervals of 3 weeks. After SIV challenge, three of these Vif/Nef-vaccinated an-

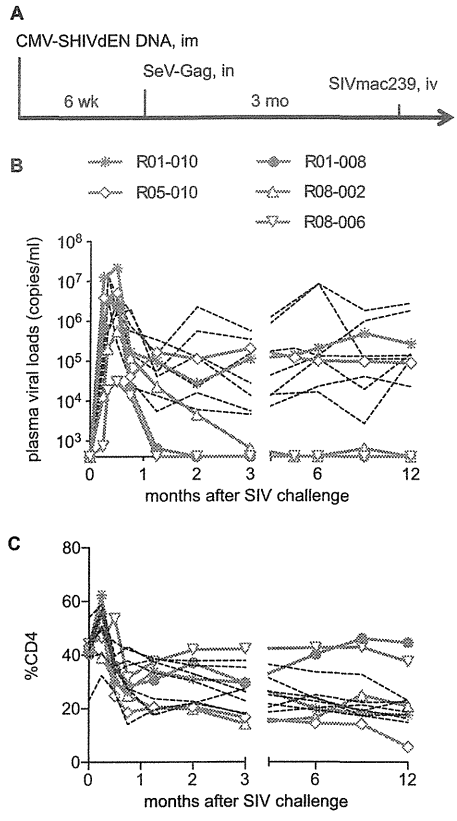

FIG 1 Viral loads and percentages of CD4 in Gag-vaccinated animals after SIVmac239 challenge. (A) Protocol of Gag vaccination and SIVmac239 challenge. (B) Plasma viral loads (SIV *gag* RNA copies/ml plasma) determined as described previously (5). The lower limit of detection is approximately 4×10^2 copies/ml. (C) Percentages of $CD4^+$ T cells in PBMCs. In panels B and C, data on unvaccinated animals ($n = 7$) are shown by dotted lines for comparison. Data on six unvaccinated (39) and two Gag-vaccinated (R01-010 and R01-008) (42) animals used in our previous studies are included.

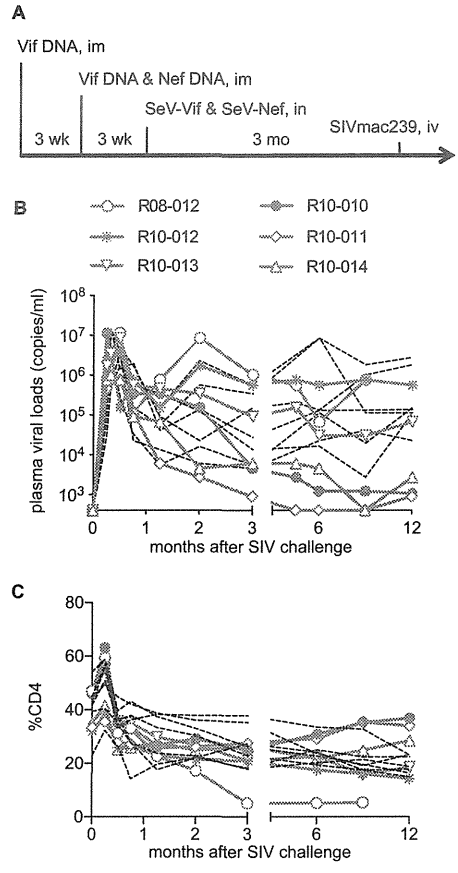

FIG 2 Viral loads and percentages of CD4 in Vif/Nef-vaccinated animals after SIVmac239 challenge. (A) Protocol of Vif/Nef vaccination and SIVmac239 challenge; (B) plasma viral loads; (C) percentages of $CD4^+$ T cells in PBMCs. In panels B and C, data on unvaccinated animals are shown by dotted lines for comparison.

imals failed to control viral replication and had high levels of set-point viral loads equivalent to those in unvaccinated macaques, but the remaining three showed SIV control with low levels of set-point viral loads (geometric mean of viral loads from 6 months to 1 year in each controller, $<2.0 \times 10^3$ copies/ml) and maintained higher CD4 levels (Fig. 2). Indeed, these six SIV controllers, consisting of three Gag-vaccinated and three Vif/Nef-vaccinated animals, showed significantly higher percentages of CD4 at 1 year than those in the remaining noncontrollers (see Fig. S1 in the supplemental material).

Gag-, Vif-, and Nef-specific $CD8^+$ T-cell responses in unvaccinated and vaccinated animals. We examined Gag-, Vif-, and Nef-specific $CD8^+$ T-cell responses in these animals. Unvaccinated macaques showed SIV-specific $CD8^+$ T-cell responses equivalent to those observed in Indian rhesus macaques (8) (Fig. 3). All of these E^+ unvaccinated macaques elicited immunodominant Nef-specific $CD8^+$ T-cell responses, consistent with our previous study analyzing other E^+ macaques (50). Gag-specific and Vif-specific $CD8^+$ T-cell responses were detected but were not immunodominant in these animals.

In contrast, all Gag-vaccinated E^+ macaques showed Gag-specific $CD8^+$ T-cell responses after the SeV-Gag boost and in the early phase after SIV challenge (Fig. 3). In these animals, Nef-specific $CD8^+$ T-cell responses mostly became immunodominant in the later phase. Importantly, all three animals that controlled SIV replication showed efficient Gag-specific $CD8^+$ T-cell responses in the acute phase postchallenge, suggesting a significant contribution of these Gag-specific $CD8^+$ T-cell responses to SIV control.

In the second group of Vif/Nef-vaccinated E^+ animals, analysis of Gag-specific, Vif-specific, and Nef-specific $CD8^+$ T-cell responses showed different patterns of responses between SIV controllers and noncontrollers (Fig. 3). In the acute phase after SIV challenge, the noncontrollers (R08-012, R10-012, and R10-013) elicited immunodominant Nef-specific $CD8^+$ T-cell responses, whereas the controllers (R10-010, R10-011, and R10-014) showed immunodominant Vif-specific $CD8^+$ T-cell responses. This suggests that the Vif-specific $CD8^+$ T-cell responses contributed to primary SIV control. In the chronic phase, Nef-specific $CD8^+$ T-cell responses were immunodominant except for one noncontroller, R10-012.

Thus, among 18 E^+ animals, consisting of seven unvaccinated, five Gag-vaccinated, and six Vif/Nef-vaccinated animals, three Gag-vaccinated and three Vif/Nef-vaccinated animals controlled SIV replication. Comparison between these six SIV controllers and the remaining 12 noncontrollers showed no significant difference in the sum of Gag-, Vif-, and Nef-specific $CD8^+$ T-cell fre-