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-γ^+ 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 $p < 0.05$.

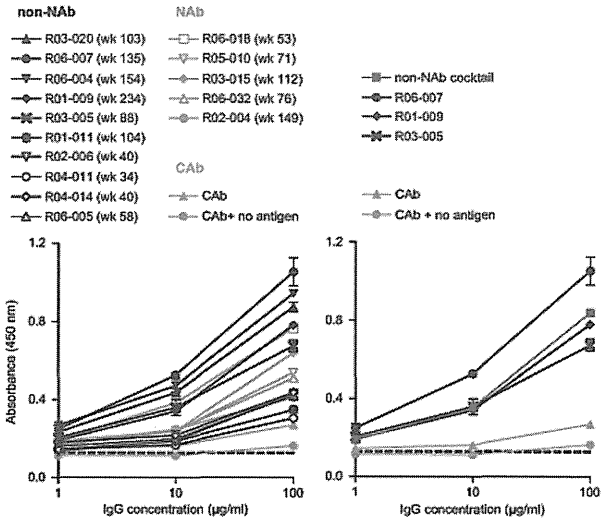

Figure 1. Binding properties of IgGs to SIV virions. Polyclonal IgGs purified from macaque plasma were subjected to whole virus ELISA using purified SIV_{mac239} virions as the antigen. Results on ten IgG lots derived from ten macaques without detectable neutralizing activity (non-NAbs; black lines), 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.

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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

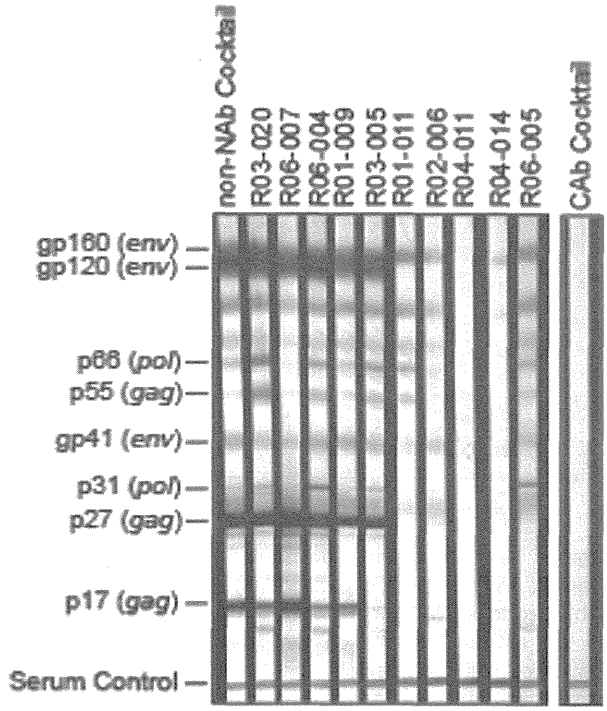

Figure 2. Binding properties of IgGs to SIV antigens. The non-NAb cocktail, ten non-NAb IgG lots derived from ten macaques, and CAb were subjected to immunoblotting (ZeptoMetrix). A representative result from two experiments is shown.

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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

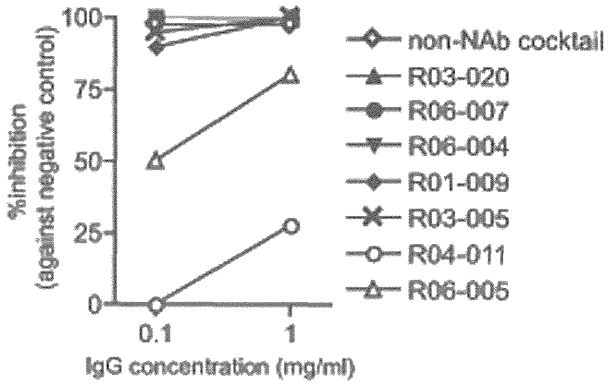

Figure 3. ADCVI activity of the non-NAb cocktail and non-NAb IgG lots. The reduction in SIV p27 concentration in the supernatant from SIV-infected cell culture with non-NAbs compared to that without antibodies is shown. A representative result, means of duplicate samples, from two experiments is shown.

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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

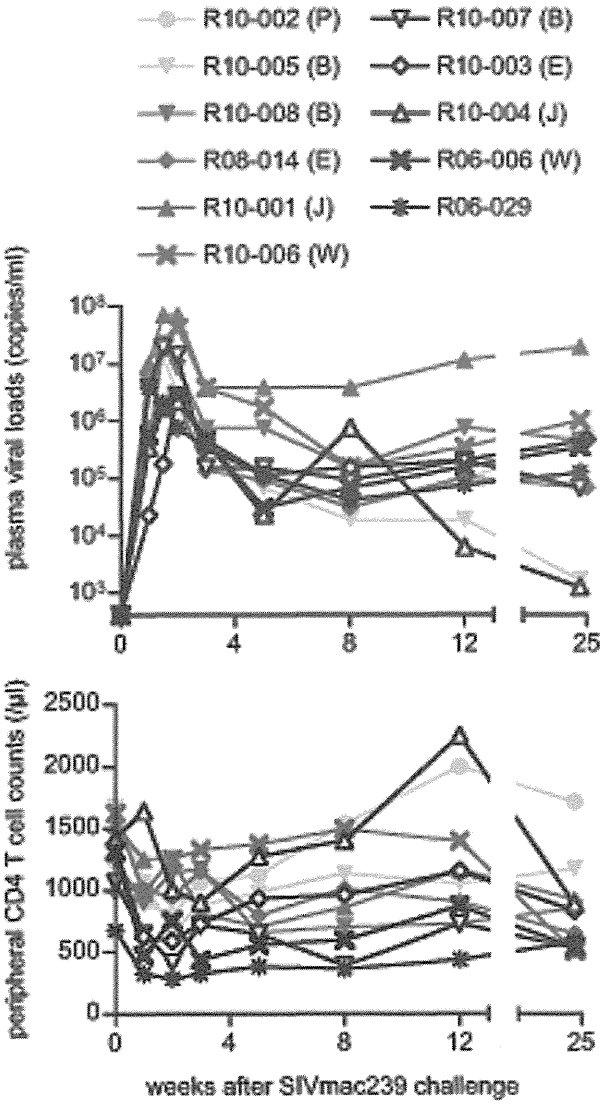

Figure 4. Passive non-NAb immunization in SIV infection. Upper panel: plasma viral loads after SIV_{mac239} challenge (SIV RNA copies/ml in plasma) in two unimmunized (green lines), four control IgG-immunized (blue), and five non-NAb-immunized macaques (black). Viral loads were determined as described 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

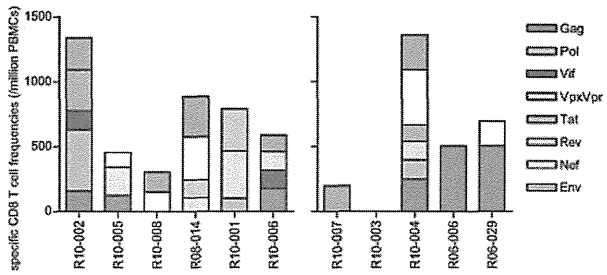

Figure 5. SIV antigen-specific $CD8^+$ T-cell responses. SIV Gag-, Pol-, Vif-, Vpx/Vpr-, Tat-, Rev-, Nef-, and Env-specific $CD8^+$ T-cell responses were measured by detection of antigen-specific IFN-γ induction using PBMCs at weeks 26-30 post-challenge.

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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.

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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

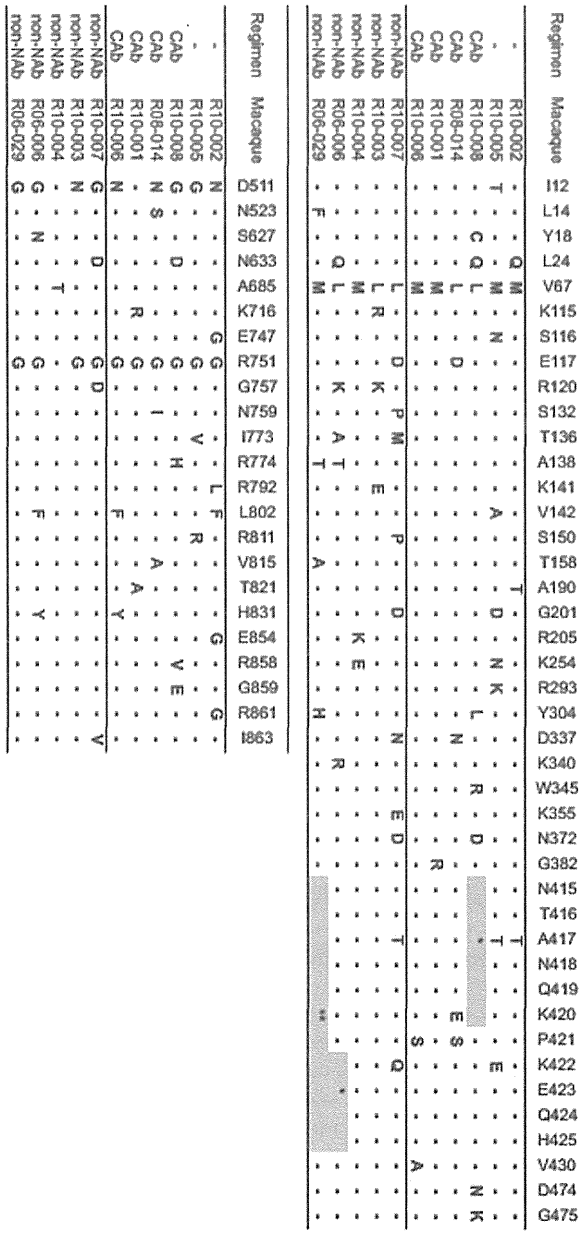

Figure 6. Predominant nonsynonymous env mutations. Viral cDNAs encoding Env were amplified from plasma RNAs obtained at 7-9 months (R10-001, R10-004, and R06-029) or 12 months (other animals) and subjected to sequencing analysis. Amino acid substitutions are shown. The asterisk (*) represents a deletion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

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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*.

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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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Generation of a replication-competent chimeric simian-human immunodeficiency virus carrying *env* from subtype C clinical isolate through intracellular homologous recombination

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ABSTRACT

A new simian-human immunodeficiency virus (SHIV), carrying *env* from an uncloned HIV-1 subtype C clinical isolate (97ZA012), was generated through intracellular homologous recombination, a DNA repair mechanism of the host cell. PCR fragments amplified from an existing SHIV plasmid (a 7-kb fragment from the 5′ end and a 1.5-kb fragment from the 3′ end) and a 4-kb fragment amplified from 97ZA012 cDNA containing *env* were co-transfected to human lymphoid cells. The resulting recombinant was subjected to serial passage in rhesus peripheral blood mononuclear cells (RhPBMCs). The resulting SHIV 97ZA012 was replication competent in RhPBMCs and monkey alveolar macrophages, and possessed CCR5 preference as an entry co-receptor. Experimental infection of rhesus macaques with SHIV 97ZA012 caused high titers of plasma viremia and a transient but profound depletion of $CD4^+$ T lymphocytes in the lung. Animal-to-animal passage was shown to be a promising measure for further adaptation of the virus in monkeys.

Introduction

Human immunodeficiency virus (HIV) infections have been a major global public health issue since their initial recognition in the 1980s. Globally, approximately 33 million individuals are living with HIV, 1.8 million people die of HIV-related complications, and 2.6 million people newly acquired the virus in 2009 (UNAIDS, 2010). Establishment of effective preventive measures is urgently needed to control the epidemic.

Extensive genomic diversity is a characteristic trait of HIV. HIV type 1 (HIV-1), the major genotype of the virus, comprises four subgroups: M, N, O, and P. Subgroup M further comprises numerous subtypes and circulating recombinant forms (CRFs), which are recombinant viruses among subtypes. Among the subtypes, subtype C plays a leading role in the epidemic, accounting for nearly 50% of global HIV infections (Hemelaar et al., 2011). Greater numbers of viral particles are detected in the vaginal secretions of pregnant individuals infected with subtype C than from persons infected with subtypes A or D (John-Stewart et al., 2005), potentially making subtype C more transmissible than others and rendering it predominant in the current epidemic. A compact V1/V2 loop and threonine at 316 located in the V3 loop of Env, distinct features shared by many subtype C isolates, may contribute to preferential replication of these viruses in the genital tract (Walter et al., 2009).

The humoral immune reaction directed against subtype C virus is unequal to that directed against subtype B virus. Virus-neutralizing antibodies mounted in individuals infected with subtype C are directed against the alpha-2 helix in the Env C3 region. This region is rarely immunogenic in subtype B virus infection (Moore et al., 2008), indicating a conformational difference in Env between these subtypes. The development of a tractable animal model for subtype C is thus necessary to establish a strategy for effective induction of neutralizing antibodies directed against the protein of this particular subtype.

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Simian-human immunodeficiency virus (SHIV) carrying Env derived from subtype C would be an especially vital tool because it would allow for evaluation of the effectiveness of vaccine-induced immunity in the context of virus infection *in vivo*.

However, only a few subtype C SHIVs are available, and none reproducibly replicates to high titers and induces disease in monkeys. In addition, limited numbers of SHIVs utilize the CCR5 molecule as an entry co-receptor. The scarcity of available SHIV strains is attributed to the difficulty in generating an infectious chimeric virus. SHIVs have been generated through recombinant DNA techniques involving implantation of a chunk of genes, such as *tat*, *rev*, *vpu*, and *env*, from the molecular clone of parental HIV-1 into the backbone of the SIV239 molecular clone. This method does not always lead to successful generation of infectious SHIV. Two presumable reasons may explain this difficulty: (1) incompatibility of a particular clone from the parental HIV-1 swarm with the SIV backbone and (2) inadequate employment of "breakpoints," sites of recombination, for the given parental clones of HIV-1 and SIV. The generation of SHIVs by the conventional technique (*i.e.*, recombination of HIV-1 genes from a molecular clone verified to be infectious to human cells with an SIV backbone at breakpoints that are reasonably assumed to be appropriate) may represent a major bottleneck for the development of new SHIV strains.

Intracellular homologous recombination (IHR) is a cellular mechanism for the restoration of DNA double-strand breaks. It also takes place when exogenously introduced DNA fragments share "homologous sequences" (Srinivasan et al., 1989). The mechanism has been attributed to the generation of infectious HIV-1 particles from cell lines carrying multiple defective provirus genomes (Inoue et al., 1991). IHR also causes generation of infectious HIV-1 through co-transfection of truncated viral cDNA clones into the cells (Kalyanaraman et al., 1988; Srinivasan et al., 1989) or through recombination between exogenous sequences and integrated chromosomal HIV sequences (Clavel et al., 1989; Srinivasan et al., 1989). It is then utilized as a measure to readily generate recombinant HIV-1 (Cheng-Mayer et al., 1990; Hertogs et al., 1998; Kellam and Larder, 1994).

We reasoned that generation of SHIV through IHR could circumnavigate the above-mentioned issues and accelerate the process as follows: (1) DNA fragments prepared by polymerase chain reaction (PCR) with cDNA from an uncloned HIV-1 genome would provide a continuum of heterogeneous sequences that potentially contain competent clone(s) in the context of infection in monkey cells *in vitro* and monkeys *in vivo* when combined with an SIV backbone, and (2) random occurrence of IHR within "homologous sequences" would likely produce multiple SHIV genomes with breakpoints at various sites, increasing the chance for emergence of a virus with favorable fitness. In addition, co-transfection of DNA fragments into cells susceptible to viral infection would subject the generated recombinant virus to multi-round replication, causing selection/evolution of a replication-competent virus. Based on this reasoning, we embarked on IHR-mediated generation of SHIV to investigate the utility of these potential advantages.

Results

Generation of recombinant virus through IHR

To generate a novel SHIV carrying the *env* gene derived from a clinical isolate of subtype C HIV-1 through IHR, we prepared three DNA fragments by PCR as depicted in Fig. 1. Approximately 1100 bps of overlapping sequence (where IHR was expected to take place) were shared by Fragments I and III, 1400 bps were shared by Fragments II-a and III, and 1200 bps were shared by Fragments II-b and III (Fig. 1).

Although we envisioned that recombination between the two DNA fragments could theoretically take place at any base within these overlaps, potentially resulting in generation of multiple sets of recombinant genomes, only replication-competent recombinant(s) would emerge as representative following transfection with these fragments into susceptible cells for lentiviral replication and multi-round replication cycles. To test this hypothesis, mixtures of Fragments I, II-a, and III (Transfection #1) or Fragments I, II-b, and III (Transfection #2), 0.2 μg of each DNA preparation, were co-transfected to human T-lymphoid cell line C8166-CCR5 cells. The cultures were maintained for 3 weeks to monitor emergence of recombinant virus by microscopic observation because the parental HIV-1 97ZA012, which contributed the *env* gene to the transfection, was known to induce syncytia in the cells (data not shown). Transfection #1 produced syncytia on day

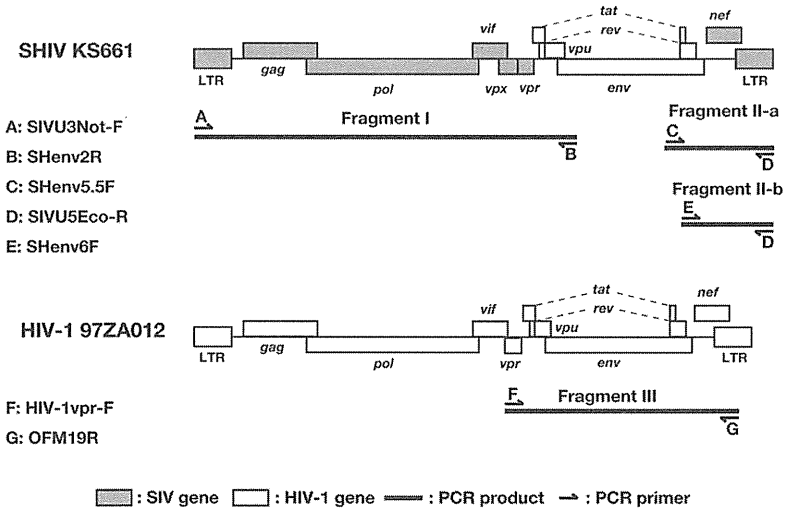

Fig. 1. Schematic representation of HIV-1/SHIV genome organizations and PCR fragments employed for co-transfection. Filled boxes represent genes derived from SIV. Open boxes represent genes derived from HIV-1. SHIV KS661, existing SHIV, carries *tat*, *rev*, *vpu*, and *env* genes from subtype B HIV-1 89.6. Broad lines represent PCR fragments; Fragments I and II-a/II-b were amplified using plasmid DNA of SHIV KS661 as a template. Fragment III was amplified from cDNA of the HIV-1 97ZA012 genome as a template. Arrows represent PCR primers whose identifiers are depicted in the figure.

14 post-transfection, and Transfection #2 caused syncytia on day 15. A small portion of each culture was collected on the day of syncytia emergence and co-cultivated with uninfected C8166-CCR5 cells to determine whether the syncytia would spread. Both initiated robust syncytia formation within 24 h post co-cultivation, suggesting generation of a recombinant virus capable of replicating in C8166-CCR5 cells. Culture supernatants collected at 3 days post-co-culture, *viz.* 17 days post-Transfection #1 and 18 days post-Transfection #2, were combined and used as starting material for *in vitro* selection/adaptation.

In vitro passage of the recombinant virus

Because we aimed to generate a new SHIV strain to be used in a macaque model, which requires a virus with the capacity to replicate to a high titer, we first subjected the "syncytium-inducing agent," which emerged through IHR, to sequential passage in rhesus macaque PBMCs (RhPBMC). We envisioned that a certain population of the recombinant carrying a suitable genotype from the parental virus swarm and appropriate recombination breakpoints might outgrow and/or evolve through the passage. Newly generated recombinant viruses are known in general to be less replication competent, especially in RhPBMCs. Such viruses have replicated better when inoculated into $CD4^+$ cell-enriched RhPBMCs by deletion of $CD8^+$ cells (Chen et al., 2000; Kamada et al., 2006). Although the viral agent was robustly replicating in human C8166-CCR5 cells, we followed the previous observations and inoculated 100 μl filtered culture supernatant of the cells into 2×10^6 rhesus macaque $CD4^+$ cell-enriched RhPBMCs (Passage #1). Virus replication was monitored by virion-associated reverse transcriptase (RT) activity released in the culture supernatant (Fig. 2).

Passage #1 revealed that the virus indeed replicated in $CD4^+$ cell-enriched RhPBMCs (Fig. 2A). The virus replicated to an initial peak of RT activity on day 3 with 2000 cpm/μl supernatant equivalent, and replication was then reduced somewhat on day 4 (1250 cpm), likely because of the addition of fresh $CD4^+$ cell-enriched PBMCs. Replication increased again to 2170 cpm on day 5, the highest RT activity in this passage. The RT activity was maintained at a high level during the subsequent 2 days (1900–2000 cpm), then declined rapidly on day 8. Cryopreserved culture supernatant (50 μl) collected from day 5 of Passage #1 was inoculated to 2×10^6 newly prepared $CD4^+$ cell-enriched RhPBMCs to confirm the observed replication property of the virus (Passage #2). Although replication in Passage #2 took longer than that in Passage #1, the Passage #2 virus also replicated to a high titer, 2180 cpm/μl supernatant equivalent on day 8, followed by a sharp decline on day 9 (1250 cpm; Fig. 2B).

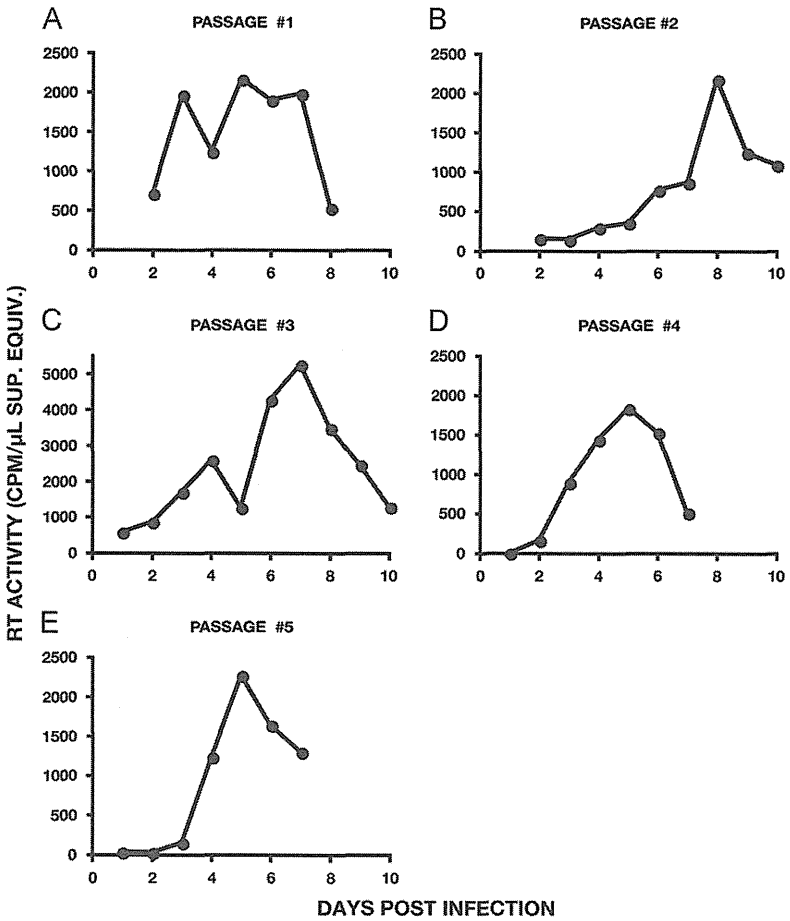

Fig. 2. Passage of the recombinant virus through RhPBMCs. Recombinant virus that emerged from C8166-CCR5 cells co-transfected with PCR fragments was serially passed through RhPBMCs (A–E). A small amount of supernatant was collected from each culture daily, and virion-associated reverse transcriptase (RT) activity was assessed. RT activities were represented as radioactive counts per minute (cpm) induced by 1 μl culture supernatant equivalent. $CD4^+$ cell-enriched RhPBMCs were employed for Passages #1–3 (A–C), and whole RhPBMCs were utilized for Passages #4 and #5 (D and E). Filtered culture supernatant was used to pass virus from existing culture to new culture, except from Passage #2 to #3, in which the mixture of cells and supernatant was inoculated to freshly prepared cells.

Because the virus appeared to reproducibly replicate in $CD4^+$ cell-enriched RhPBMCs, we next examined whether the virus could replicate in unmanipulated RhPBMCs, a cell preparation without enrichment of $CD4^+$ cells, by taking a small portion of the ongoing culture on day 8 and co-cultivating it with fresh whole RhPBMCs (Passage #3). For this experiment, we monitored the RT activity of the culture supernatant from Passage #2 daily and decided to set up Passage #3 because of a substantial increase in RT activity on day 8. Passage #3 resulted in robust virus replication that reached 5,250 cpm/μl supernatant equivalent on day 7, then declined during the following days, suggesting that it possessed replication capacity in unmanipulated RhPBMCs (Fig. 2C). To determine whether we could reproduce this observation in a more rigorous setting, culture supernatant without cells, collected on day 7 from Passage #3, was inoculated into whole RhPBMCs newly prepared from normal rhesus macaques (Passage #4). The virus replicated robustly to a high titer, reaching 1850 cpm/μl supernatant equivalent on day 5, followed by a rapid decline (Fig. 2D). To confirm the observation, we inoculated supernatant collected on day 5 of Passage #4 into another RhPBMC preparation without manipulation a second time (Passage #5). The virus reproducibly replicated in primary monkey cells, with a peak of RT activity on day 5 post-infection (2280 cpm; Fig. 2E). We concluded that a new recombinant virus capable of replicating in RhPBMCs was generated/evolved through IHR/*in vitro* passage. We designated the culture supernatant collected on day 5 from Passage #5 as SHIV 97ZA012 and subjected it to further characterization.

Genomic organization of SHIV 97ZA012

To elucidate the genomic organization of SHIV 97ZA012, the nt sequence was determined on cDNA that was reverse-transcribed from virion-associated viral genomic RNA prepared from the culture supernatant. The obtained sequence was compared with those of SHIV KS661 and HIV-1 97ZA012 (Fig. 3). Genomic analysis revealed that the 5′ and 3′ breakpoints were at the 282nd nt of *env* gp120, upstream of the V1/V2 loop, and the 756th nt of the *env* gp41 cytoplasmic domain, respectively (Fig. 3A). The breakpoints that gave rise to replication-competent virus were not necessarily at the interface of genes juxtaposed to each other. This is also the case in many CRFs of HIV-1, examples of naturally occurring recombinants (Carr et al., 2001; Guimaraes et al., 2008; Koulinska et al., 2001; Perez et al., 2006; Piyasirisilp et al., 2000).

SimPlot (Lole et al., 1999) analysis revealed that the genomic fragment derived from HIV-1 97ZA012 did not completely match

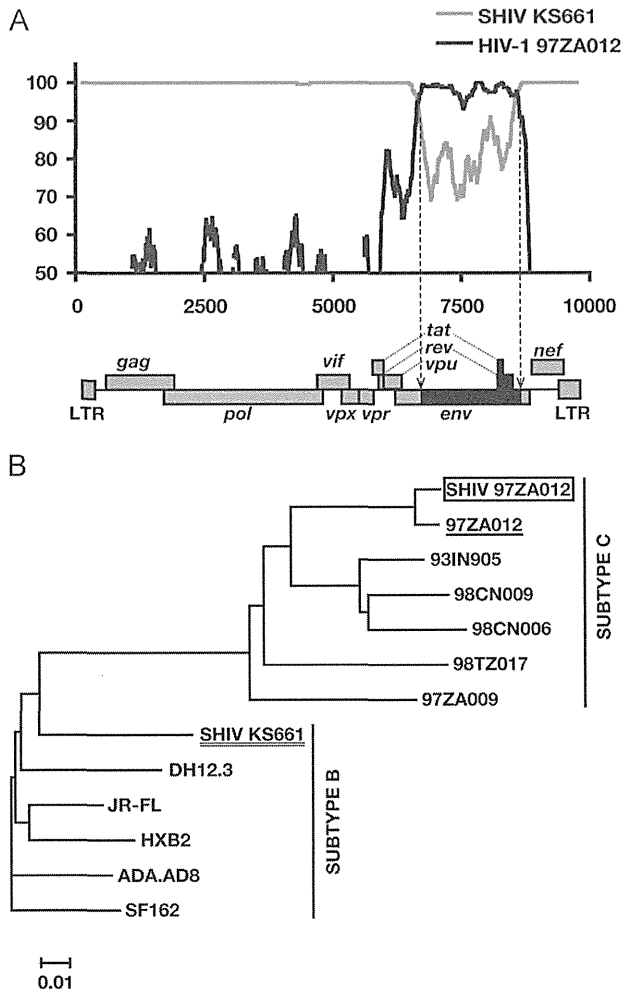

Fig. 3. Genomic organization of SHIV 97ZA012. (A) Breakpoints in SHIV 97ZA012 were analyzed by the SimPlot program with SHIV KS661 (gray) and HIV-1 97ZA012 (black) as references. Schematic SHIV genome organization was aligned to similarity plot for a visual purpose. (B) Phylogenetic analysis of SHIV 97ZA012 *env* portion. The gene portion identified in (A), between nt 6429 and 8325 (in HIV-1 HXB2) and derived from HIV-1 97ZA012, was subjected to phylogenetic analysis with the corresponding sequence of the reference virus isolates. The phylogenetic tree was generated by the neighbor-joining method.

the registered sequence of the virus (GenBank Accession No. AF286227). To ascertain that the recombinant virus indeed carries the *env* sequence derived from HIV-1 97ZA012, we conducted a phylogenetic analysis of the sequence with the corresponding region of HIV-1 strains belonging to subtype B or C, including the registered sequence of 97ZA012 (Fig. 3B). The supposed 97ZA012-derived sequence naturally formed a subcluster with the registered sequence and was positioned in the cluster of subtype C strains in the phylogenetic tree. We concluded that the observed difference in sequences between SHIV 97ZA012 and the registered HIV-1 97ZA012 was within the extent of quasispecies of a single isolate.

Replication properties of SHIV 97ZA012 in C8166-CCR5 cells and RhPBMCs

To characterize the replication properties of SHIV 97ZA012, we inoculated the virus into human C8166-CCR5 cells and RhPBMCs. Because HIV-1 is unable to replicate in monkey cells, the relative replication capability of the newly generated virus to the parental HIV-1 97ZA012 was assessed in C8166-CCR5 cells. SHIV KS661, which provided the backbone for SHIV 97ZA012, was also examined. In RhPBMCs, replication of SHIV 97ZA012 was compared with that of SIV239, the most widely used SIV. The viruses were normalized to an infectious unit (multiplicity of infection [MOI]=0.01 or 0.1 median tissue culture infective dose [$TCID_{50}$/cell]) and inoculated into these cells. Replication of the viruses was monitored by virion-associated RT activity in the culture supernatant (Fig. 4). Because C8166-CCR5 cells were highly susceptible to HIV-1/SIV and progressed to cell death, infections at a higher multiplicity (MOI=0.1) resulted in lower peak RT activities compared with those at a lower multiplicity (Fig. 4A). Regardless of multiplicity, SHIV 97ZA012 exhibited a slower replication kinetic compared with that of the parental HIV-1 97ZA012, likely due to the chimeric structure of the virus (Li et al., 1992; Shibata et al., 1991) or to the result of adaptation to monkey cells. SHIV KS661 also exhibited faster replication kinetics compared with SHIV 97ZA012.

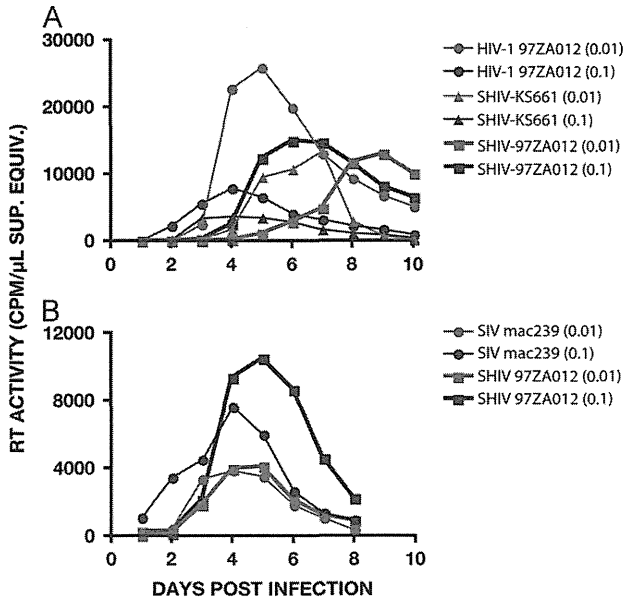

Fig. 4. Replication of SHIV 97ZA012 in C8166-CCR5 cells (A) and RhPBMCs (B). The replication property of SHIV 97ZA012 in these cells was compared with those of HIV-1 97ZA012, SHIV KS661 (A) and SIV239 (B). The viruses were normalized by the infectious unit (MOI=0.01 and 0. 1 TCID50/cell) and spinoculated to these cells at 1200 × *g* for 60 min. Gray symbols/lines represent virus replication at MOI=0.01, and black symbols/lines represent virus replication at MOI.=0.1. Culture supernatant was collected daily, and virion-associated reverse transcriptase activities were assessed.

While SHIV 97ZA012 exhibited a slower replication profile compared with the reference viruses in C8166-CCR5 cells, replication of SHIV 97ZA012 in RhPBMCs was comparable with that of SIV239 (Fig. 4B). Infection at a MOI=0.01 resulted in somewhat more production of progeny in SIV239 than in SHIV 97ZA012 during the first 3 days. When the experiment was set up at a MOI=0.1, SIV239 produced markedly more progeny than did SHIV 97ZA012 during the first 3 days of infection, while SHIV 97ZA012 replicated to higher titers than did SIV239 at day 4 and thereafter. To estimate the total production of progeny virus during the observation period, the areas under the curve (AUC) of each virus were compared. The AUC of SHIV 97ZA012 was approximately 1.5-fold greater than that of SIV (data not shown). Based on these data, we concluded that SHIV 97ZA012 is as replication competent as SIV239 in rhesus macaque PBMCs.

Co-receptor preference of SHIV 97ZA012

The parental HIV-1 97ZA012 strain reportedly utilizes CCR5 as an entry co-receptor (Broder and Jones-Trower, 1999). The chimeric structure of gp120 carried by SHIV 97ZA012 (between KS661, which was originally derived from HIV-1 89.6, and 97ZA012) and uncertainty of the co-receptor preference of 97ZA012 Env in the context of the SHIV/macaque cell system prompted us to subject SHIV 97ZA012, along with control viruses, to a co-receptor usage assay. Each virus, normalized by an infectious unit, was inoculated to RhPBMCs in the presence of 5 μM AMD3100 (a small-molecule CXCR4 inhibitor), AD101 (a small-molecule CCR5 inhibitor), or both. Virus replication was monitored for 7 days by virion-associated RT activity release in the culture supernatant (Fig. 5). SIV239, which has been established as a CCR5-utilizing virus, replicated to a high titer in the absence of any inhibitor (Fig. 5A). AMD3100 had little impact on the replication of the virus, as described previously (Zhang et al., 2000). In the presence of AD101 or both inhibitors, however, replication of SIV239 was substantially impaired. In contrast, SHIV KS661, which has been reported to predominantly utilize CXCR4 as an entry co-receptor (Matsuda et al., 2010), exhibited a replication profile opposite to that of SIV239: no impairment of replication in the presence of AD101, but remarkably delayed replication in the presence of AMD3100, and complete suppression in the presence of both inhibitors (Fig. 5B). When control viruses exhibited the replication profiles described above, SHIV 97ZA012 exhibited a replication profile similar to that of SIV239: no impact on replication in the presence of AMD3100, but complete suppression when AD101 was present in the culture (Fig. 5C). Based on these results, we concluded that SHIV 97ZA012 is a CCR5-utilizing virus, as is the parental HIV-1 97ZA012 in human cells.

Replication of SHIV 97ZA012 in macrophages

Many CCR5-utilizing HIV-1 strains replicate in monocyte-derived macrophages, which is a biological property called "macrophage tropism." Macrophage tropism has been shown to be associated with viral neurotropism (Gorry et al., 2001), a subject to be investigated in non-human primate AIDS models. To clarify the biological property of the virus in macrophages, SHIV 97ZA012, along with macrophage-tropic and non-macrophage-tropic viruses, normalized by RT activity was inoculated to rhesus macaque primary alveolar macrophage (RhAM) cultures prepared from three uninfected animals. Virus replication was monitored by virion-associated RT activity released in

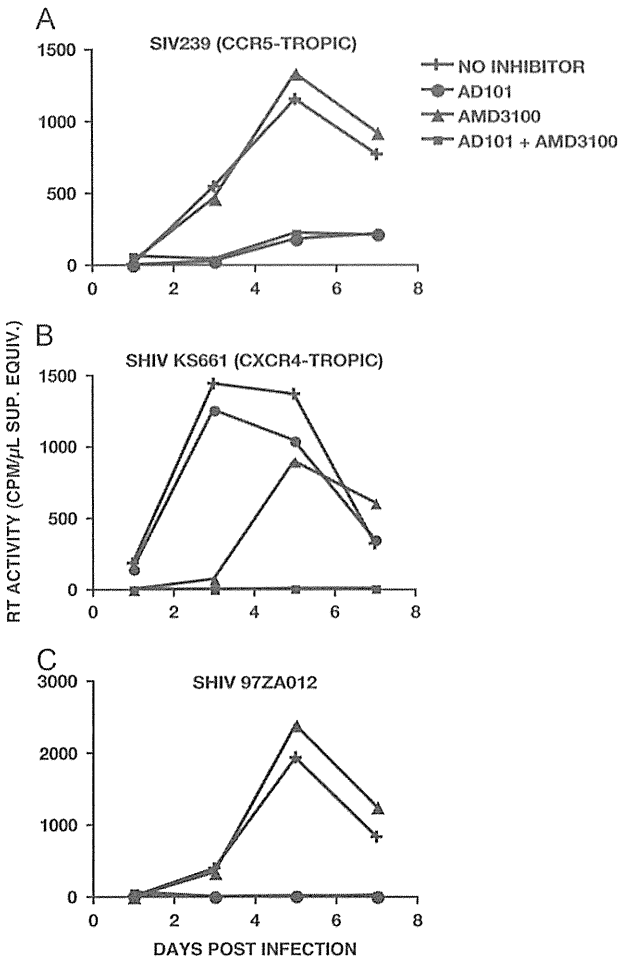

Fig. 5. Co-receptor usage of SHIV 97ZA012. SHIV 97ZA012, along with CCR5-tropic SIV239 and predominantly CXCR4-tropic SHIV KS661, was inoculated into RhPBMCs in the presence of small-molecule co-receptor inhibitor(s) (5 μM), and replication was assessed by reverse transcriptase activity in the culture supernatant for 7 days. The following compounds were utilized as co-receptor inhibitors: AMD3100 for anti-CXCR4 inhibitor and AD101 for anti-CCR5 inhibitor, which was kindly provided by Dr. Julie Strizki, Schering-Plough Research Institute, Kenilworth, NJ.

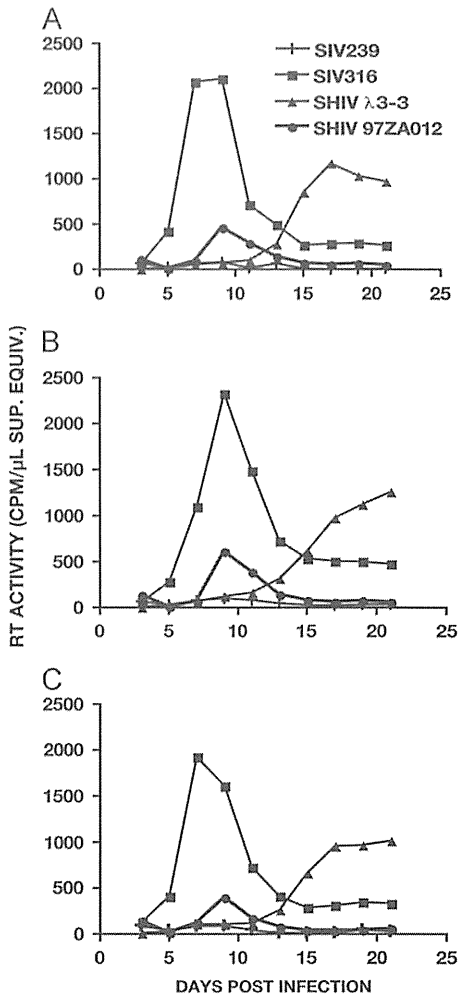

Fig. 6. Replication of SHIV 97ZA012 in primary rhesus alveolar macrophages (RhAMs). SHIV 97ZA012, along with macrophage-tropic SIV316 and SHIV λ3-3 and non-macrophage-tropic SIV239, was inoculated into primary RhAMs, and its replication was monitored for 3 weeks. RhAMs were prepared from three animals independently (A–C). Virus replication was assessed by reverse transcriptase activity in the culture supernatant.

the culture supernatant (Fig. 6). Overall, the viruses exhibited consistent replication profiles among cell cultures prepared from different individuals (Fig. 6A–C). SIV316, a macrophage-tropic variant of SIV239 (Desrosiers et al., 1991), replicated to the highest titers among the viruses, reaching a peak around days 7–9 post-infection (2000–2500 cpm/μl). SHIV λ3-3, a macrophage-tropic SHIV (Igarashi et al., 2007), exhibited a delayed replication profile and reached titers of >1000 cpm/μl after 17 days post-infection. In contrast, SIV239, which is reportedly incapable of replicating in cells of this type (Mori et al., 1993), produced no measurable RT activity in the supernatant during the observation period. Under this condition, SHIV 97ZA012 replicated productively, although not as robustly as SIV316 or SHIV λ3-3, and reached peak virus replication on day 9 post-infection (400–600 cpm/μl). Based on these results, we concluded that SHIV 97ZA012 is macrophage tropic.

Experimental infection of rhesus macaques with SHIV 97ZA012

Biological properties of the newly generated SHIV 97ZA012 revealed in the study, including CCR5 utilization, a robust replication profile in RhPBMCs, and infectiousness in primary macrophages, justified experimental infection of monkeys with the virus. Prior to the infection, we prepared an ample volume of animal challenge stock of the virus by inoculating SHIV 97ZA012 seed to RhPBMCs. Culture supernatant was collected daily and assessed for virus replication by RT activity. Fractions of culture supernatant collected on days 8 and 9 that exhibited the highest RT activities (2450 and 2550 cpm/μl supernatant equivalent) were combined, filtered through a 0.45-μm membrane, divided into aliquots designated SHIV 97ZA012 animal challenge stock, and stored in liquid nitrogen. The infectious titer of the virus stock was 1.51×10^4 $TCID_{50}$/ml, and retention of its preference for CCR5 as an entry co-receptor was verified (data not shown).

The animal challenge stock of SHIV 97ZA012, 1×10^5 $TCID_{50}$, was intravenously inoculated into each of three rhesus macaques. Virus replication was monitored by viral RNA load in plasma samples that were collected periodically (Fig. 7). The virus replicated to substantially high titers in all three animals, reaching an initial peak of 1.03×10^8 copies/ml for MM533, 4.52×10^6 copies/ml for MM535, and 1.83×10^8 copies/ml for MM536 at week 1.1 (day 8) post-inoculation (Fig. 7A). After the initial peak,

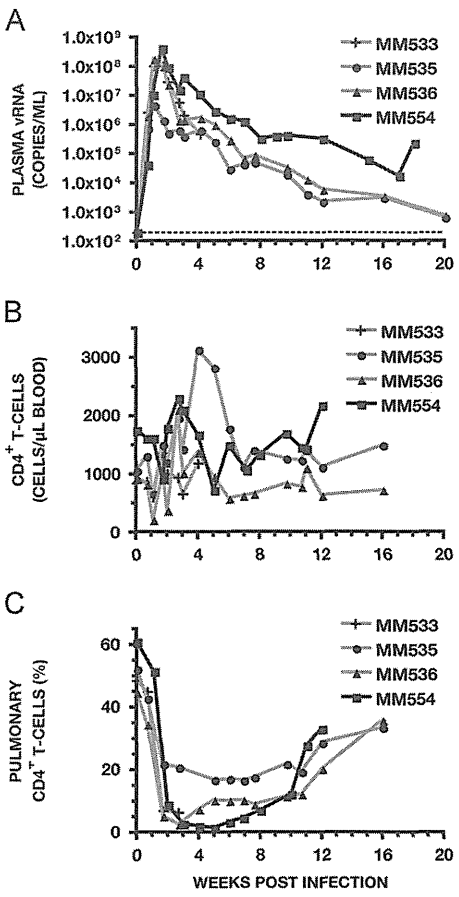

Fig. 7. Experimental infection of rhesus macaques with SHIV 97ZA012. 1×10^5 TCID50 SHIV 97ZA012 animal challenge stock was intravenously inoculated into three rhesus macaques, MM533, MM535, and MM536. Their plasma viral burdens (A), circulating $CD4^+$ T lymphocytes (B), and $CD4^+$ T cells in the alveolar space (C) were monitored periodically. Lymph node cells and whole blood collected from MM535 and MM536 at week 10.7 were transferred to MM554 (A–C).

viral burdens of all three animals declined somewhat, but remained at approximately 1×10^6 copies/ml between weeks 3 and 5. One of the animals, MM533, was found to be lethargic and subsequently died at week 4. The cause of death was not related to primate lentivirus infection. The plasma viral burdens of the remaining two animals gradually declined from week 6 onward, resulting in barely detectable levels at week 20 (650 copies/ml for MM535 and 740 copies/ml for MM536).

(Fig. 7B). Although all three animals experienced a transient decrease in cell numbers, the cell numbers promptly rebounded thereafter and basically stabilized after week 8 for MM535 and week 6 for MM536.

SIV and HIV-1 preferentially replicate at an "effector site," such as the mucosal tissues of the genital organs, lung, and gastrointestinal tract, where CCR5-positive effector memory $CD4^+$ T lymphocytes, the primary viral target cells, predominantly reside. Here, they cause substantial depletion of cells during the acute phase of infection (Brenchley et al., 2004; Okoye et al., 2007; Veazey et al., 1998; Veazey et al., 2003). Because SHIV 97ZA012 utilizes the CCR5 molecule as an entry coreceptor, it was envisaged that the virus depletes effector memory $CD4^+$ T lymphocytes as do SIV and HIV-1. We examined the fluctuation in $CD4^+$ T lymphocytes in the pulmonary space of SHIV 97ZA012-infected animals as the representative effector site because the procedure causes the least severe insult to animals, allowing us to conduct frequent monitoring. It is known that SIV239 infection results a substantial depletion of pulmonary $CD4^+$ T cells in infected rhesus macaques, along with depletion of the cells in the gastrointestinal tract (Okoye et al., 2007). In contrast to the cells in circulation, $CD4^+$ T cells in the alveolar space exhibited a substantial decline during the acute phase of SHIV 97ZA012 infection (Fig. 7C). The percentages of cells dropped from 48.5%, 52.1%, and 44.7% for MM533, MM535, and MM536, respectively, on day 0 to 6.3% for MM533 at week 2.7, 16.8% for MM535 at week 5, and 2.6% for MM536 at week 2.7. The alveolar $CD4^+$ T lymphocytes remained at low levels in the animals until week 11, then gradually increased toward pre-infection levels. Based on the results described above, it was concluded that SHIV 97ZA012 robustly replicated during the acute phase of infection, causing remarkable reduction of $CD4^+$ T cells in the alveolar space. However, the animals eventually controlled the virus replication. SHIV 97ZA012 thus appeared to be less likely to cause disease in monkeys.

Multiple reports on the evolution of primate lentivirus through animal-to-animal passage have shown that initially less-efficiently replicating and non-pathogenic viruses transform to replication-competent and highly pathogenic viruses (Joag et al., 1996; Reimann et al., 1996a; Sharma et al., 1992). We applied this strategy to SHIV 97ZA012. At week 10.7, the axillary lymph nodes were collected from animals MM535 and MM536. Cells prepared from the lymph nodes from both animals, 5×10^8 cells in total, were resuspended with 10 ml anti-coagulated whole blood collected simultaneously from these monkeys. The resuspension was intravenously transferred to another rhesus macaque, MM554. The virological and immunological parameters of the recipient animal were monitored as described above. The virus induced viremia with an initial peak of 4.2×10^8 copies/ml at week 1.6, followed by a gradual decrease until week 8, then was maintained at approximately 3–4 $\times 10^5$ copies/ml for 4 weeks (Fig. 7A). Although the plasma viral burden of MM554 declined after the initial peak, its titer was constantly higher than those inoculated with the original animal challenge stock of SHIV 97ZA012, indicating likely improvement in virus replication. Numbers of circulating $CD4^+$ T lymphocytes in MM554 did not change substantially compared with the other animals (Fig. 7B). MM554 manifested a more substantial reduction in alveolar $CD4^+$ T cells than did the other three animals (Fig. 7C). The percentage of $CD4^+$ T lymphocytes dropped from 61% at day 0 to 2.8% at week 3, and further declined to 1.4% at week 5. The cell numbers remained low until week 7 and started to recover thereafter. Based on these results, we concluded that animal-to-animal passage appears to have made SHIV 97ZA012 more fit to replicate in macaque monkeys, warranting improvement by further passage.

Discussion

In this study, we successfully generated a new SHIV strain carrying Env derived from an HIV-1 subtype C primary isolate, HIV-1 97ZA012, utilizing IHR. The presumable advantages of the method employed in the current study over conventional methods utilizing existing/newly generated restriction sites are random utilization of breakpoints within homologous sequences and selection of replication-competent recombinants through multi-round replication in the susceptible cells. These factors may have contributed to the generation of the new SHIV in the current study.

Initially, SHIVs have been generated through recombination of infectious molecular clones of SIV and HIV-1 (Shibata et al., 1991 #143; Li et al., 1992 #141; Luciw et al., 1995 #152; Reimann

et al., 1996b #153). The availability of HIV-1 infectious molecular clone(s) was thus a prerequisite for the generation of SHIV. The SHIVs generated in the abovementioned manner generally exhibited insubstantial replication profiles *in vitro* and *in vivo* (Shibata et al., 1991 #143; Li et al., 1992 #141; Sakuragi et al., 1992 #151; Luciw et al., 1995 #152; Reimann et al., 1996b #153}. Plasmid clones carrying open reading frames that were derived from PCR fragments amplified from HIV-1 provirus were subsequently employed as the source of HIV-1 genes, instead of DNA fragments excised from full-length molecular clones (Chen et al., 2000 #1; Kuwata et al., 2002 #135}. Kuwata et al. generated 30 SHIV clones representing Env protein from three independent isolates of HIV-1 from the initial exertion to generate SHIV strains representing six separate HIV-1 isolates. Of 30 clones, three were infectious to human cells, and only one productively replicated in monkey PBMCs but exhibited only modest replication *in vivo* {Kuwata et al., 2002 #135}. Hence, generation of replication-competent SHIVs by the conventional method is inefficient.

In contrast, the IHR-mediated method described in the current study generated replication-competent SHIV 97ZA012 without the requirement for an infectious molecular clone of the parental HIV-1, exploration of appropriate restriction sites, or examination of each plasmid clone for infectivity. This was performed in a considerably shorter time frame in our experience, saving several months compared with conventional methods. However, one would argue that IHR-mediated generation of SHIV does not allow for a detailed genetic analysis, such as mutagenesis of particular gene(s), because of the virus being "swarm." While this is undeniable, the vast majority of currently available replication-competent SHIVs are resultants of evolution through animal-to-animal passage and exist as quasispecies (Joag et al., 1996 #49; Reimann et al., 1996a #52; Igarashi et al., 1999 #156; Harouse et al., 2001 #154; Song et al., 2006 #158; Nishimura et al., 2010 #157}. A molecular-cloned virus representing the properties of the swarm is attainable by introduction of consensus sequences to a molecular clone, if necessary.

Co-transfection of genome fragments into C8166-CCR5 cells appears to have generated multiple recombinant viruses with distinct breakpoints and/or *env* genes. Following short-term propagation of the virus that emerged after co-transfection, viral genomic RNA from culture supernatant was subjected to sequencing without a cloning step. We were unable to determine the sequence within the overlaps between Fragments I and III or II-a/b and III (Fig. 1) because of multiple sequence peaks at each location, suggesting the existence of multiple DNA templates (data not shown). The mixture of recombinants was substantially "purified" through serial passages in RhPBMCs (Fig. 2), allowing us to determine breakpoints (Fig. 3). This observation supports the relevance of the concept employed in the current study; that is, selection of replication-competent recombinants through multi-round replication.

The selected replication-competent recombinant virus possesses breakpoints within the *env* gene, resulting in a "mosaic" *env* structure. Because primate lentiviruses encode multiple genes in different reading frames in a stratified fashion, *tat* and *rev* genes also became mosaic. The mosaic *tat*, *rev*, and *env* genes are not uncommon among circulating recombinant forms of HIV-1 (Carr et al., 2001; Koulinska et al., 2001; Ng et al., 2012; Su et al., 2000; Yamaguchi et al., 2008), although these breakpoints are less likely to be employed in rational construction of SHIVs.

SHIV 97ZA012 replicated to a titer comparable with that of SIV239 in RhPBMCs, and the observed trend was unaffected by the reduction in MOI (from 0. 1 to 0.01 in Fig. 4B), an indication of the replication competence of SHIV 97ZA012 in the cells. In addition, SHIV 97ZA012 exhibited productive replication in rhesus primary alveolar macrophages, although not as robust as that of SIV316 (Fig. 6). In our experience, not every "macrophage-tropic" virus replicates in alveolar macrophages. While SIV 251 and SIVsmE543 have been reported to be macrophage-tropic in monocyte-derived macrophages (Hirsch et al., 1997; Miller et al., 1998), they did not replicate in alveolar macrophages (Igarashi et al., unpublished). Alveolar macrophages of human or rhesus macaque express miniscule amounts of CD4 and CCR5 (Mori et al., 2000; Worgall et al., 1999). Therefore, SHIV 97ZA012 may be able to gain entry to cells expressing minimal numbers of receptors/coreceptors, as is SIV316 (Puffer et al., 2002). Whether "CD4-independence" is a property shared by many subtype-C Env or is specific to 97ZA012 or whether a "mosaic" Env protein between HIV-1 89.6 and 97ZA012 caused this notable property remains to be investigated.

This study also presents potential shortcomings of IHR to be resolved in the future. The first drawback is that only a limited variety of Env may function in the C8166-CCR5 cells utilized in the current study, resulting in generation of SHIVs reflecting this potential restriction. Prior to the current study, we inoculated nine primary isolates of HIV-1 subtype C obtained from the National Institutes of Health (NIH) AIDS Research & Reference Reagent Program into the cells and found that seven of them replicated in the cells with syncytia (data not shown). Cells susceptible to a broad range of HIV-1 primary isolates should be utilized in the future. The second shortcoming is the relatively long (2-week) "incubation phase" following co-transfection. The low frequency of recombination events and less-efficient DNA transfection may have been responsible for the elongated incubation period. Upregulation of IHR through certain means, such as overexpression of Rad51 (Vispe et al., 1998) (an enzyme that plays an important role in IHR) prior to co-transfection of viral cDNA fragments or utilization of certain cells that are shown to exhibit elevated IHR activity (e.g., breast cancer cells (Mao et al., 2009)) could augment the efficiency of recombination. To achieve higher transfection efficiency, utilization of well-established and highly transfectable cells, such as 293 T (formerly 293tsA1609neo) cells (DuBridge et al., 1987), followed by co-cultivation with cells susceptible to virus replication, such as PBMCs, should be explored.

We were able to detect syncytia formation in the culture transfected with DNA fragments only after 2 weeks. Once syncytia emerged, however, the virus replicated productively in C8166-CCR5 cells and subsequently in RhPBMCs enriched with $CD4^+$ cells and unmanipulated RhPBMCs through a passage in RhPBMCs. SHIV 97ZA012 evolved to be replication competent in RhPBMCs and infectious to RhAMs. The virus may have been replication competent in monkey cells from the beginning. Although HIV-1 97ZA012, which contributed *env* to SHIV 97ZA012, may have been predisposed to be adequate as a parental virus for generation of SHIV, we have the impression that the method we employed in this study generated a pool of recombinants and selected suitable one(s) through *in vitro* passage. As mentioned above, the recombinant virus that initially emerged was a mixture, and the final genotype(s) was selected and/or evolved through *in vitro* passage. The only traits of HIV-1 97ZA012 of which we were aware were its replication competence in human PBMCs and its preference for CCR5 as an entry co-receptor to the cells (data not shown).

SHIV 97ZA012 reproducibly replicated to high titers *in vivo* with a major reduction in pulmonary $CD4^+$ T cells during the acute phase of infection. Considering the paucity of available SHIV strains carrying subtype C Env and a CCR5 co-receptor preference in the field, the virus generated in the current study would immediately fit the interest for evaluation of anti-subtype C vaccine candidates. The virus would be especially useful when the efficacy of antiviral interventions is judged by reduction of the

initial peak viral load or prevention of virus-induced depletion of $CD4^+$ T lymphocytes in the effector sites.

Although we successfully generated an SHIV strain competent in tissue culture, the generation of a proficient virus *in vivo* remains arduous. Indeed, SHIV 97ZA012 replicated to substantially high titers during the acute phase of infection. However, the plasma viral load waned with time, as did SHIV strains generated previously. Animal-to-animal passage would augment its replication *in vivo*, as we attempted in this study.

This study has demonstrated the versatility of IHR-mediated generation of SHIV. The method enables utilization of a PCR fragment amplified from uncloned virus as a source for SHIV. This method can be further extended to generate SHIV strains with sequences amplified from clinical samples, such as patient plasma, to strengthen the panel of challenging viruses for evaluation of an anti-HIV vaccine.

Conclusions

By employing IHR, a replication-competent SHIV carrying Env derived from a CCR5-tropic, subtype C HIV-1 97ZA012 strain was generated.

Materials and methods

Cells

C8166-CCR5 cells from a human T-lymphotropic virus type-1-transformed human T-lymphoid cell line that was transduced to express human CCR5 and established as described previously (Soda et al., 1999) were generously provided by Dr. Hiroo Hoshino, Gunma University, Japan. The cells were cultured in Roswell Park Memorial Institute 1640 medium (Life Technologies Corporation, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 4 mM L-glutamine, and 2 mM sodium pyruvate (*R*-10). RhPBMCs were prepared and cultured as described previously (Imamichi et al., 2002), with minor modification; 160 units/ml recombinant interleukin-2 (Wako Pure Chemicals, Osaka, Japan) was added to the medium to maintain lymphocytes. RhAMs were collected through a bronchoalveolar lavage technique and cultured as described previously (Imamichi et al., 2002).

Viruses

Virus stocks of SIV239 (Kestler et al., 1988), SIV316 (Desrosiers et al., 1991), SHIV DH12R CL7 (Sadjadpour et al., 2004), and SHIV λ3-3 (Igarashi et al., 2007) were propagated in RhPBMCs following inoculation with transfection supernatant of the proviral plasmid of each virus. Dr. Ronald C. Desrosiers at Harvard University kindly provided the plasmid of SIV239. Dr. Malcolm A. Martin at the National Institute of Allergy and Infectious Diseases (NIAID), NIH, generously contributed the plasmid of SIV316 with permission from Dr. Desrosiers and the plasmids of SHIV DH12R CL-7 and SHIV λ3-3. Infectious titers ($TCID_{50}$) of the virus stocks were determined by titration as described previously (Shibata et al., 1997), with a minor modification; the indicator cells employed for titration were RhPBMCs in this study. The HIV-1 97ZA012 isolate was obtained from The UNAIDS Network for HIV Isolation and Characterization through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, and was propagated briefly in human PBMCs after acquisition.

Preparation of cDNA from HIV-1 97ZA012 genomic RNA

Culture supernatant was harvested from human PBMCs infected with HIV-1 97ZA012 on day 8 post-inoculation. Virion-associated RNA was extracted with the QIAamp Viral RNA mini kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. The extracted RNA was subsequently subjected to synthesis of cDNA with Super Script III (Life Technologies Corporation) following the manufacturer's instructions. For the reaction, the following primer was utilized: OFM19-R (5′-aggcaagctttattgaggctta-3′ at 9604-9625 in HIV-1 HXB2).

Generation of recombinant virus through IHR

Two segments of the SHIV genome (Fragments I and II; Fig. 1) were amplified by PCR with pSHIV KS661, an infectious molecular clone of SHIV C2/1 (Shinohara et al., 1999), as a template. Positions of PCR primers were numbered relative to the SIV239 or HIV-1 HXB2 genome sequence (GenBank accession nos. M33262 and K03455, respectively). For Fragment I, the following primer pair was employed: SIVU3Not-F forward primer (5′-atgcggccgctggaaggatttattacagtgcaag-3′, at 1–25 in SIV239) and SHenv2R rear primer (5′-cacagagtggggttaattttacac-3′, at 6580–6603 in HIV-1 HXB2). Two sets of Fragment II (II-a and II-b) were amplified by PCR. For Fragment II-a, the following primer pair was used: SHenv5.5F forward primer (5′-tcataatgatagtaggaggc-3′, at 8278–8297 in HIV-1 HXB2) and SIVU5Eco-R rear primer (5′-tgcagaattctgctagggatttcctgcttcggtt-3′, at 10255–10279 in SIV239). For Fragment II-b, the following primer pair was utilized: SHenv6F forward primer (5′-gcggagcctgtgcctcttcagc-3′, at 8504–8525 in HIV-1 HXB2) and SIVU5Eco-R rear primer. A segment of the HIV-1 97ZA012 genome containing *env* and flanking genes (Fragment III) was amplified through PCR with cDNA of viral genomic RNA as a template. For amplification of Fragment III, the following primer pair was applied: HIVvpr-F forward primer (5′-agatggaacaagccccagaaga-3′ at 5557–5578 in HIV-1 HXB2) and OFM19-R rear primer.

All PCR reactions were conducted with the Expand Long Range dNTPack (Roche Diagnostic Corporation, Basel, Switzerland) under the following conditions: initial denaturation at 94 °C for 2 min, followed by 10 cycles of amplification consisting of denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, and extension at 68 °C for 8 min. The reaction was continued with 25 cycles of amplification consisting of denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, and extension at 68 °C for 8 min (plus 20 s at every cycle), followed by a final extension at 68 °C for 7 min.

Mixtures of Fragments I, II-a, and III (Transfection #1) or I, II-b, and III (Transfection #2), 0.2 μg of each fragment, were co-transfected into C8166-CCR5 cells through diethylaminoethyl-dextran-mediated DNA uptake followed by osmotic shock (Takai and Ohmori, 1990). After co-transfection, the cell cultures were maintained in 24-well plates at 37 °C and monitored by daily microscopic observation. On day 14 for Transfection #1 and day 15 for Transfection #2, a small portion of each culture was taken and independently co-cultured with uninfected C8166-CCR5 cells for an additional 3 days.

In vitro passage of recombinant virus

RhPBMCs were prepared as described above. The $CD8^+$ cell fraction was removed from the cell preparation using phycoerythrin (PE)-conjugated anti-CD8 antibody (clone SK1; BD BioSciences, San Jose, CA, USA) and anti-PE-conjugated magnetic microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), following the manufacturers' instructions.

Culture supernatant from C8166-CCR5 cells co-cultured with Transfection #1 or Transfection #2 on days 17 and 18 post-transfection, respectively, was mixed and filtered through a 0.45-μm membrane. The supernatant, 100 μl in total, was subsequently inoculated into 2×10^6 $CD4^+$ cell-enriched RhPBMCs (Passage #1). Freshly prepared $CD4^+$ cell-enriched RhPBMCs, 2×10^6 cells in total, were added to Passage #1 on day 3 post-inoculation. Virus replication was assessed by virion-associated RT activity in the culture supernatant, as described below. Cryopreserved supernatant on day 5 (50 μl) from Passage #1 was thawed and inoculated into 2×10^6 $CD4^+$ cell-enriched RhPBMCs (Passage #2). On day 3, freshly prepared $CD4^+$ cell-enriched RhPBMCs, 2×10^6 cells in total, were added to Passage #2. The RT activity of the supernatant was monitored daily, and a small portion of Passage #2 on day 8, when the RT activity rose substantially, was subsequently co-cultured with 2×10^6 freshly isolated RhPBMCs without enrichment of $CD4^+$ cells (Passage #3). On day 3, 2×10^6 freshly prepared $CD4^+$ cell-enriched RhPBMCs were added to Passage #3. Cryopreserved supernatant from Passage #3 on day 7 (50 μl) was thawed and inoculated into 2×10^6 freshly prepared RhPBMCs without manipulation (Passage #4). On day 3, 2×10^6 freshly prepared RhPBMCs without manipulation were added to Passage #4. Cryopreserved supernatant from Passage #4 on day 5 (50 μl) was thawed and inoculated into 2×10^6 freshly prepared RhPBMCs without manipulation (Passage #5). Fresh RhPBMCs without manipulation were added to Passage #5 on day 3.

Reverse transcriptase assay

Virion-associated RT activity in the culture supernatant was evaluated as described previously (Willey et al., 1988), with a minor modification; α-^{32}P TTP was purchased from PerkinElmer Inc. (Waltham, MA, USA) in this study.

Genomic analysis of the recombinant virus

Virion-associated viral genomic RNA was extracted from culture supernatant collected on day 5 of Passage #5 and reverse transcribed, as described above. For sequencing, the bulk cDNA was directly subjected to the Sanger dideoxy method with a BigDye Terminator Cycle Sequencing Kit (Life Technologies Corporation) and analyzed with an ABI PRISM 3130 Genetic Analyzer (Life Technologies Corporation).

Breakpoints of the recombinant virus were determined through comparison of sequences of the recombinant virus with those of SHIV KS661 (identical to SHIV C2/1, GenBank accession no. AF217181) and p97ZA012 (GenBank accession no. AF286227). The sequences were aligned using Clustal X software (Thompson et al., 1997) and analyzed using SimPlot software (Lole et al., 1999), with a window size of 250 bp and a step size of 20 bp.

A portion of the nt sequence of SHIV 97ZA012, at nt 6429–8325 (in HIV-1 HXB2) and derived from HIV-1 97ZA012, was subjected to phylogenetic analysis with the corresponding sequence of the following reference virus isolates: 93IN905 (GenBank accession no. AY669742), 98CN009 (AF286230), 98CN006 (AF286229), 98TZ017 (AF286235), 97ZA009 (AY118166), and 97ZA012 (AF286227) for subtype *C* references, and DH12.3 (AF069140), JR-FL (U63632), ADA.AD8 (AF004394), SF162 (EU123924), HXB2 (K03455), and SHIV KS661 for subtype *B* references. Phylogenetic analysis by neighbor-joining method (Saitou and Nei, 1987) was conducted using Clustal X software. The analyzed result was plotted by Mega 5 software (Tamura et al., 2011).

Replication kinetics of SHIV 97ZA012

Virus stocks subjected to comparison were normalized to the infectious titer (MOI=0.01 or 0.1 $TCID_{50}$/cell). For infection of M8166-CCR5 cells, HIV-1 97ZA012, SHIV KS661, and SHIV 97ZA012 were titrated using TZM-bl cells, which were granted from the NIH AIDS Research & Reference Reagent Program. For infection of RhPBMCs, SIV239 and SHIV 97ZA012 were titrated in RhPBMCs. The virus stocks were inoculated to C8166-CCR5 cells or RhPBMCs by spinoculation (O'Doherty et al., 2000) at $1200 \times g$ for 60 min.

After inoculation, culture supernatant was replaced daily with freshly prepared medium and stored at −20 °C until analysis of its RT activity. The AUC of the replication kinetics, an estimate of the total production of progeny virus during the observation period, was calculated for each virus using the Prism 4 Software (GraphPad Software, Inc., La Jolla, CA, USA).

Co-receptor usage assay

The co-receptor preference of SHIV 97ZA012 on RhPBMCs was analyzed as described previously (Matsuda et al., 2010), with the modifications described below. Infectious titers of SHIV 97ZA012, SIV239, and SHIV DH12R CL7 were normalized by the infectious titer (MOI=0.03 $TCID_{50}$/cell) in this study. In the presence of 5 μM of the small-molecule co-receptor inhibitors AMD3100 (De Clercq et al., 1994) (Sigma-Aldrich, St. Louis, MO, USA), AD101 (Trkola et al., 2002), or both, the viruses were spinoculated ($1200 \times g$ for 60 min) into RhPBMCs, and virus replication was monitored for 7 days. During the experiment, culture supernatant was replaced on days 1, 3, and 5 with freshly prepared culture medium containing the same concentration of corresponding inhibitor(s). Culture supernatant was collected on days 1, 3, 5, and 7 and stored at −20 °C until assessment of RT activity. Dr. Julie Strizki, Schering-Plough Research Institute, Kenilworth, NJ, generously provided the AD101.

Replication of SHIV 97ZA012 in RhAMs

RhAMs were collected and cultured as described above. SHIV 97ZA012, SIV239, SIV316, and SHIV λ3-3 were normalized by RT activity (MOI=17 cpm equivalent/cell) and spinoculated ($1200 \times g$ for 60 min) to 5×10^5 cells in a 24-well plate. Culture supernatant was replaced every other day with freshly prepared medium and stored at −20 °C until assessment of its RT activity.

Experimental infection of rhesus monkeys with SHIV 97ZA012

Rhesus macaques of Indian origin, approximately 4 kg in body weight, were used for experimental infection with SHIV 97ZA012. Phlebotomy, bronchoalveolar lavage, lymph node biopsy, and virus inoculation were conducted under anesthesia by intramuscular injection of a mixture of ketamine chloride (Ketalar; Daiichi Sankyo, Tokyo, Japan) at 5–10 mg/kg and xylazine chloride (Celactal; Bayer Healthcare, Leverkusen, Germany) at 1.5–2.0 mg/kg. Animals 533, 535, and 536 were intravenously inoculated with 1×10^5 $TCID_{50}$ SHIV 97ZA012. Animal 554 intravenously received a mixture of anticoagulated whole blood (10 ml) and lymph node cells (5×10^8 cells) collected from animals 535 and 536 at 10.7 weeks post-inoculation. All animal experiments were conducted in a biosafety level 3 animal facility in compliance with institutional regulations approved by the Committee for Experimental Use of Nonhuman Primates of the Institute for Virus Research, Kyoto University, Kyoto, Japan.

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A Novel Protective MHC-I Haplotype Not Associated with Dominant Gag-Specific CD8$^+$ T-Cell Responses in SIVmac239 Infection of Burmese Rhesus Macaques

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Abstract

Several major histocompatibility complex class I (MHC-I) alleles are associated with lower viral loads and slower disease progression in human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) infections. Immune-correlates analyses in these MHC-I-related HIV/SIV controllers would lead to elucidation of the mechanism for viral control. Viral control associated with some protective MHC-I alleles is attributed to CD8$^+$ T-cell responses targeting Gag epitopes. We have been trying to know the mechanism of SIV control in multiple groups of Burmese rhesus macaques sharing MHC-I genotypes at the haplotype level. Here, we found a protective MHC-I haplotype, *90-010-Id* (D), which is not associated with dominant Gag-specific CD8$^+$ T-cell responses. Viral loads in five D$^+$ animals became significantly lower than those in our previous cohorts after 6 months. Most D$^+$ animals showed predominant Nef-specific but not Gag-specific CD8$^+$ T-cell responses after SIV challenge. Further analyses suggested two Nef-epitope-specific CD8$^+$ T-cell responses exerting strong suppressive pressure on SIV replication. Another set of five D$^+$ animals that received a prophylactic vaccine using a Gag-expressing Sendai virus vector showed significantly reduced viral loads compared to unvaccinated D$^+$ animals at 3 months, suggesting rapid SIV control by Gag-specific CD8$^+$ T-cell responses in addition to Nef-specific ones. These results present a pattern of SIV control with involvement of non-Gag antigen-specific CD8$^+$ T-cell responses.

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Introduction

Virus-specific CD8$^+$ T-cell responses play a central role in the control of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) replication [1,2,3,4,5]. Genetic diversities of HLA or major histocompatibility complex class I (MHC-I) result in various patterns of CD8$^+$ T-cell responses in HIV-infected individuals. Cumulative studies on HIV infection have indicated the association of MHC-I genotypes with higher or lower viral loads [6,7,8,9,10]. In some MHC-1 alleles associating with lower viral loads and slower disease progression, certain CD8$^+$ T-cell responses restricted by these MHC-I molecules have been shown to be responsible for HIV control [11,12,13]. In rhesus macaque AIDS models, *Mamu-A*01*, *Mamu-B*08*, and *Mamu-B*17* are known as protective alleles, and macaques possessing these alleles tend to show slower disease progression after SIVmac251/SIVmac239 challenge [14,15,16,17].

Recent studies have indicated great contribution of CD8$^+$ T-cell responses targeting Gag epitopes to reduction in viral loads in HIV/SIV infection [18,19,20,21]. Viral control associated with some protective MHC-I alleles is attributed to Gag epitope-specific CD8$^+$ T-cell responses [22,23,24]. 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 an escape mutation with viral fitness costs, leading to lower viral loads [22,24,25,26,27]. On the other hand, CD8$^+$ T-cell responses targeting SIV antigens other than Gag, such as Mamu-B*08- or Mamu-B*17-restricted Vif and Nef epitopes, have been indicated to exert strong suppressive pressure on SIV replication [28,29,30,31,32,33]. Accumulation of our knowledge on the potential of these non-Gag-specific as well as Gag-specific CD8$^+$ T-cell responses for HIV/SIV control should be encouraged for elucidation of viral control mechanisms.