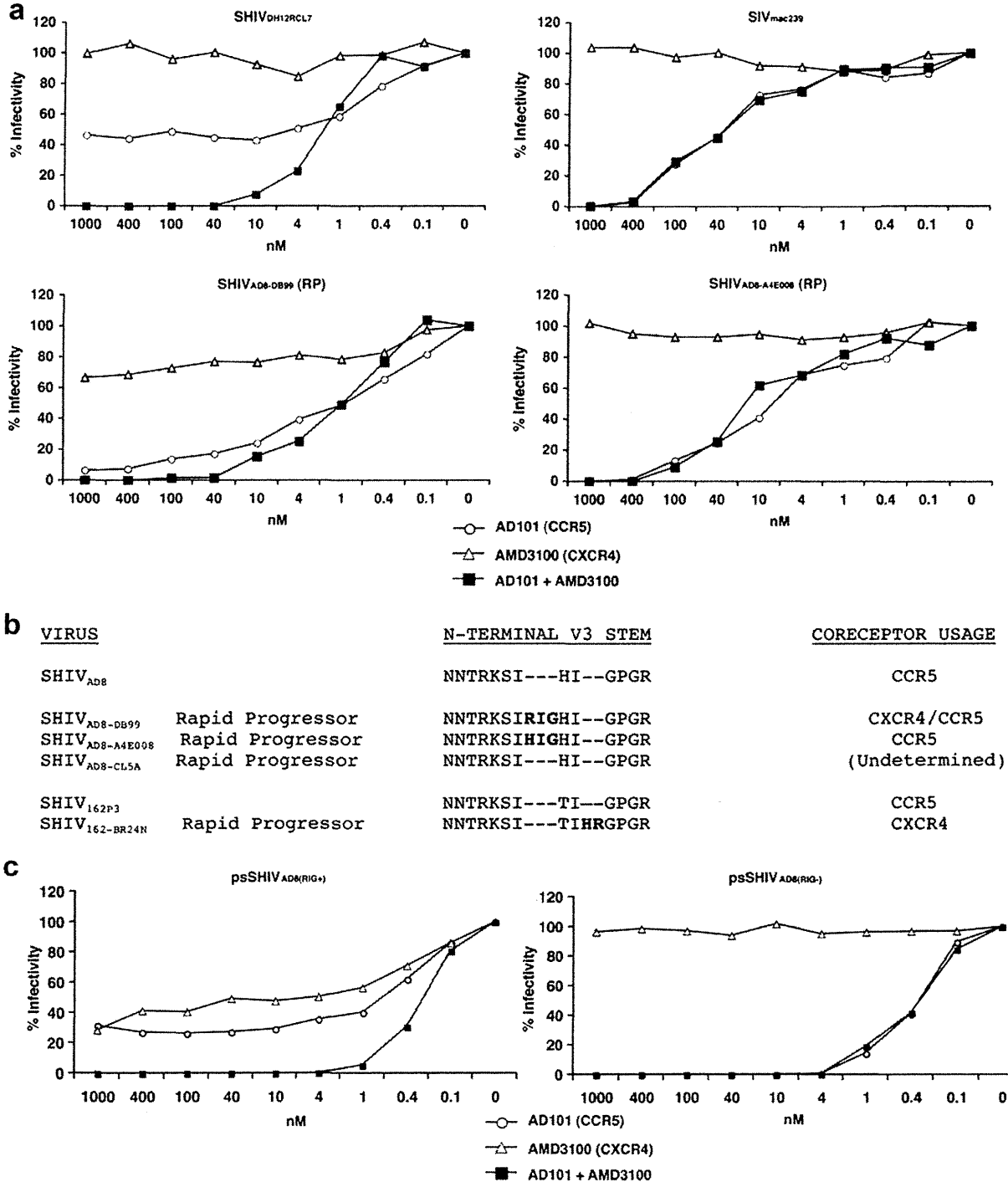

FIG. 9. Coreceptor utilization of $SHIV_{AD8}$ derivatives isolated from rapid progressors. (a) TZM-bl cells were infected in quadruplicate with viruses ($SHIV_{AD8\text{-}DB99}$ and $SHIV_{AD8\text{-}A4E008}$) recovered from rapid progressors DB99 and A4E008, respectively, in the presence of the indicated amounts of the small-molecule coreceptor inhibitors AD101 (CCR5), AMD 3100 (CXCR4), or both. SIV_{mac239} and $SHIV_{DH12RCL\text{-}7}$ were also analyzed as representative R5-tropic and dual-tropic viruses, respectively. The luciferase activities present in cell lysates 24 h p.i. were measured, and percent infectivities were determined in the absence or presence of coreceptor inhibitors. (b) gp120 sequences from the N-terminal V3 regions of $SHIV_{AD8}$ variants, recovered from three RP animals, were aligned with the starting $SHIV_{AD8}$ V3 loop. The V3 regions of the R5-tropic $SHIV_{SF162P3}$ and its $SHIV_{SF162\text{-}BR24N}$ derivative, which also emerged in an RP, are included in the alignment. (c) Coreceptor utilization of virus, pseudotyped with Envs present in RP DB99 at the time of necropsy, containing or lacking the 3-aa RIG V3 loop insertion.

TABLE 2. Clinical and pathological findings in rhesus monkeys infected with $SHIV_{AD8\#2}$ and its immediate derivatives

Animal	Clinical data/pathological findings
CJ8B	Euthanized (wk 199); uncontrolled diarrhea; wt loss
CK15	Euthanized (wk 112); *P. carinii* pneumonia
CJ58	Total $CD4^+$ T cells, 154/mm^3 (wk 111)
CE8J	Euthanized (wk 117); uncontrolled diarrhea, *C. coli* enteritis
CJ35	Total $CD4^+$ T cells, 270/mm^3 (wk 129)
CJ3V	Euthanized (wk 135); uncontrolled diarrhea; typhlocolitis
CK5G	Total $CD4^+$ T cells: 101/mm^3 (wk 101)
DB99	Euthanized (wk 23); rapid progressor
DA1Z	Total $CD4^+$ T cells, 545/mm^3 (wk 65)
A4E008	Euthanized (wk 20); rapid progressor
DA4W	Total $CD4^+$ T cells, 92/mm^3 (wk 64)
CL5A	Euthanized (wk 19); rapid progressor
CL98	Euthanized (wk 100); disseminated *M. avium*

(1.6×10^2 to 1.5×10^5 RNA copies/ml). This variability was also observed in pairs of animals inoculated with identical $SHIV_{AD8\#2}$ derivatives (*viz.* CK15 and CJ58, and DB99 and DA1Z). An extreme example of the nonlinkage between viral-RNA levels and $CD4^+$ T cell loss with $SHIV_{AD8}$ occurred with animal CK5G, which had 43 and 42 circulating naïve and memory $CD4^+$ T cells/μl, respectively, at week 86 p.i. and a plasma viral load of only 5.4×10^2 RNA copies/ml. During the chronic phase of $SHIV_{AD8}$ infections, the loss of naïve $CD4^+$ T cells was more rapid and more marked than the depletion of the memory subset, as was previously observed in SIVsmE543-infected animals (35) (Fig. 6). By week 80, for example, NPs had sustained an 87 to 93% loss of naïve $CD4^+$ T cells from their preinoculation levels, whereas the depletion of memory cells was significant, but not as pronounced. The dissociation of plasma virus loads and $CD4^+$ T cell loss is reminiscent of the previously reported infection of pig-tailed macaques with SIVl'hoest and SIVsun (4). In that study, 8 of 12 infected animals developed immunodeficiency over a 5-year period while maintaining set-point viremia between 10^2 and 10^3 RNA copies/ml.

We do not presently understand why naïve $CD4^+$ T lymphocytes are lost in $SHIV_{AD8}$ NPs. Based on coreceptor expression, this T cell subset expresses CXCR4, not CCR5, on its surface and should therefore be refractory to infection by R5-tropic SHIVs and virus-induced cell killing. An assessment of the coreceptor utilization status of late-stage viruses recovered from $SHIV_{AD8}$ NPs, in fact, revealed that a coreceptor switch had not occurred in these animals (see Fig. S2 in the supplemental material). Although a dissociation between viral-RNA levels and memory/naïve $CD4^+$ T cell loss was observed, the NPs did experience increased memory $CD4^+$ T lymphocyte turnover (see Fig. S1 in the supplemental material), even in animals with very low plasma virus loads. Activation-induced proliferation and killing of memory $CD4^+$ T cells during the lengthy chronic $SHIV_{AD8}$ infection might therefore be responsible for driving the differentiation of naïve $CD4^+$ lymphocytes into memory cells and impose an unsustainable drain on this $CD4^+$ T cell subset. It is also possible that $SHIV_{AD8}$ infection of rhesus macaques negatively affects naïve $CD4^+$ T lymphocyte homeostasis in the thymus, thereby impeding the differentiation or emigration of this T cell subset. It has also recently been reported that the loss of naive $CD4^+$ T cells during SIVsmE543 infections was associated with the presence of autoreactive antibodies to $CD4^+$ T lymphocytes, platelets, double-stranded DNA, and phospholipid (27). Increased numbers of circulating IgG-coated $CD4^+$ T cells were observed in that study, and the levels of autoreactive antibodies were correlated with the extent of naïve $CD4^+$ T cell depletion.

Approximately 20% of rhesus monkeys infected with SIVmac/SIVsm lineage viruses become RPs, experiencing persistently high virus set points, rapid and complete losses of memory $CD4^+$ T cells, undetectable or transient antiviral antibody responses, and early onset (3 to 6 months p.i.) of symptomatic disease (6). Despite losing virtually all of their memory $CD4^+$ T lymphocytes, SIV RPs, at the time of death, usually maintain preinoculation levels of naïve $CD4^+$ T cells (35). This was not the case for $SHIV_{AD8}$ RPs. Although all three experienced early and massive depletions of memory $CD4^+$ T cells, two of the infected macaques had lost virtually all of their naïve $CD4^+$ T cells at the time of euthanasia. In one of these animals (DB99), the virus recovered at the time of euthanasia, as well as a virus pseudotyped with an Env possessing the RIG insertion in the V3 loop, had acquired the capacity to infect cells expressing CXCR4 (Fig. 9a and c). Interestingly, coreceptor switching has been previously reported to occur during RP infections of macaques inoculated with a different R5-tropic SHIV, $SHIV_{SF162P3}$ (18, 19, 47). In one of the $SHIV_{SF162P3}$ coreceptor-switching events, the insertion of two positively charged amino acids (HR) immediately upstream of the V3 loop GPGR crown (Fig. 9b) was shown to confer X4 tropism (18). In the case of $SHIV_{AD8\text{-}DB99}$, a 3-aa (RIG) insertion, also located in the N-terminal V3 stem and which increased the net charge of the V3 loop from +3 to +5, was responsible for the acquisition of CXCR4 usage. The insertion of HIG at the same location of the $SHIV_{AD8\text{-}A4E008}$ V3 region did not affect the net charge and did not confer tropism for CXCR4-expressing cells.

Independent and unrecognized cross-species transmissions and spread of SIVsm at different U.S. primate facilities during the 1970s contributed to the emergence of SIVmac and SIVsmE660 lineages with distinctive replicative and pathogenic phenotypes. The serial passaging of $SHIV_{AD8}$ in rhesus monkeys described here also resulted in an AIDS-inducing primate lentivirus with its own characteristic properties. First, in contrast to commonly used pathogenic SIVs, $SHIV_{AD8\#2}$ and its immediate derivatives generated sustained but, as previously noted, highly variable set-point virus loads in NPs. Similarly variable viral loads were also observed in eight rhesus monkeys inoculated with four independent $SHIV_{AD8}$ stocks prepared from macaques CK15, CE8J, CL98, and CJ58 at the time of their euthanasia (data not shown). Profound depletions of both memory and naïve $CD4^+$ T cells, which accompany relatively low virus set points (geometric mean level, 1.7×10^3 RNA copies/ml) in NPs, is a second property that distinguishes the R5-tropic $SHIV_{AD8}$ from pathogenic SIVs. Finally, unlike SIVs, $SHIV_{AD8}$ RPs experience an initial loss of memory $CD4^+$ T lymphocytes and a later rapid deletion of naïve $CD4^+$ T cells prior to death, which in one animal occurred following a CCR5-to-CXCR4 coreceptor switch. Based on the results shown in Fig. 4 and Table 2, we plan to use and distribute

$SHIV_{AD8\#2LN}$, $SHIV_{AD8\#2PBMC}$, or the SHIVs recovered from NPs at the time of euthanasia ($SHIV_{AD8-CL98}$, $SHIV_{AD8-CK15}$, or $SHIV_{AD8-CE8J}$) as challenge viruses in vaccine experiments. Animals inoculated with cell-free preparations of the last group of viruses have experienced variable but sustained plasma viremia associated with a gradual but significant $CD4^+$ T cell loss during 30 weeks of infection. Some of these macaques have developed a rapid-progressor clinical course.

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Cutting Edge

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Cutting Edge: T Cells Monitor N-Myristoylation of the Nef Protein in Simian Immunodeficiency Virus-Infected Monkeys

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The use of the host cellular machinery is essential for pathogenic viruses to replicate in host cells. HIV and SIV borrow the host-derived N-myristoyl-transferase and its substrate, myristoyl-CoA, for coupling a saturated C_{14} fatty acid (myristic acid) to the N-terminal glycine residue of the Nef protein. This biochemical reaction, referred to as N-myristoylation, assists its targeting to the plasma membrane, thereby supporting the immunosuppressive activity proposed for the Nef protein. In this study, we show that the host immunity is equipped with CTLs capable of sensing N-myristoylation of the Nef protein. A rhesus macaque $CD8^+$ T cell line was established that specifically recognized N-myristoylated, but not unmodified, peptides of the Nef protein. Furthermore, the population size of N-myristoylated Nef peptide-specific T cells was found to increase significantly in the circulation of SIV-infected monkeys. Thus, these results identify N-myristoylated viral peptides as a novel class of CTL target Ag. *The Journal of Immunology*, **2011, 187: 608–612.**

Major histocompatability complex class I-restricted CTLs are a critical component of the host immunity, capable of detecting virus-infected cells and eliminating them to clear infection. However, pathogenic viruses often exhibit an outstanding ability to escape from CTL attack by introducing amino acid mutations in the target proteins while maintaining their proper functions. Therefore, CTL recognition of antigenic epitopes that admit of no arbitrary mutations would be crucial for efficient host defense and CTL-based vaccine development.

Recently, the repertoire of Ags recognized by T cells has been expanded to include not only proteins but also lipidic molecules. For example, it has been demonstrated that human CD1b-restricted T cells can recognize mycobacteria-derived mycolic acids and their glycosylated species, which are critical components for the cell wall architecture of mycobacteria (1). It remains to be established whether T cell responses to these lipidic Ags could significantly contribute to host defense against human tuberculosis, but their protective role has been noted in the guinea pig model of tuberculosis (2).

Viruses do not possess their own lipids, but can biosynthesize lipopeptides by using the host cellular machinery. Given that N-myristoylation is an irreversible protein modification that occurs in the conserved motifs spanning the N-terminal short stretch of several amino acid residues (3) and that N-myristoylation of the Nef protein is critical for HIV pathogenesis (4), we reasoned that N-myristoylated Nef peptides would be an ideal Ag targeted by the host CTL response. In this study, we successfully established a rhesus macaque $CD8^+$ T cell line that specifically recognized N-myristoylated peptides of the SIV Nef protein. More importantly, these N-myristoylated Nef peptide-specific T cells were found to expand significantly in SIV-infected monkeys.

Materials and Methods

Ags

Chemical reagents were purchased from Nacalai Tesque (Kyoto, Japan) unless otherwise indicated. Peptides were synthesized by a manual Fmoc solid-phase peptide synthesis technique using Wang-resin precoupled with a relevant C-terminal amino acid (EMD Chemicals, Gibbstown, NJ). Acylation was carried out by reacting the N-terminal amino group with acid anhydrides prepared with *N,N'*-diisopropylcarbodiimide, followed by release of the acylated peptides in 95% trifluoroacetic acid. Crude Ags were purified by HPLC with a gradient elution based on water and methanol with 0.1% trifluoroacetic acid. After freeze-drying, the samples were subjected to liquid chromatography mass spectrometry analysis, using a C18 column (GL Sciences) at a flow rate of 0.5 ml/min with a solvent system of water and methanol with 0.1% formic acid.

Establishment of lipopeptide-specific T cell lines

PBMCs (1.2×10^7/well) obtained from rhesus macaque monkeys were cultured with synthetic lipopeptides at a concentration of 5 μg/ml, and antigenic stimulation was repeated every 2 wk in the presence of irradiated autologous PBMCs. IL-2 was added at 0.3 nM after the second stimulation, and the concentration was gradually increased to 3 nM by the fourth stimulation. The culture medium used was RPMI 1640 (Invitrogen, Carlsbad,

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Abbreviation used in this article: PI, propidium iodide.

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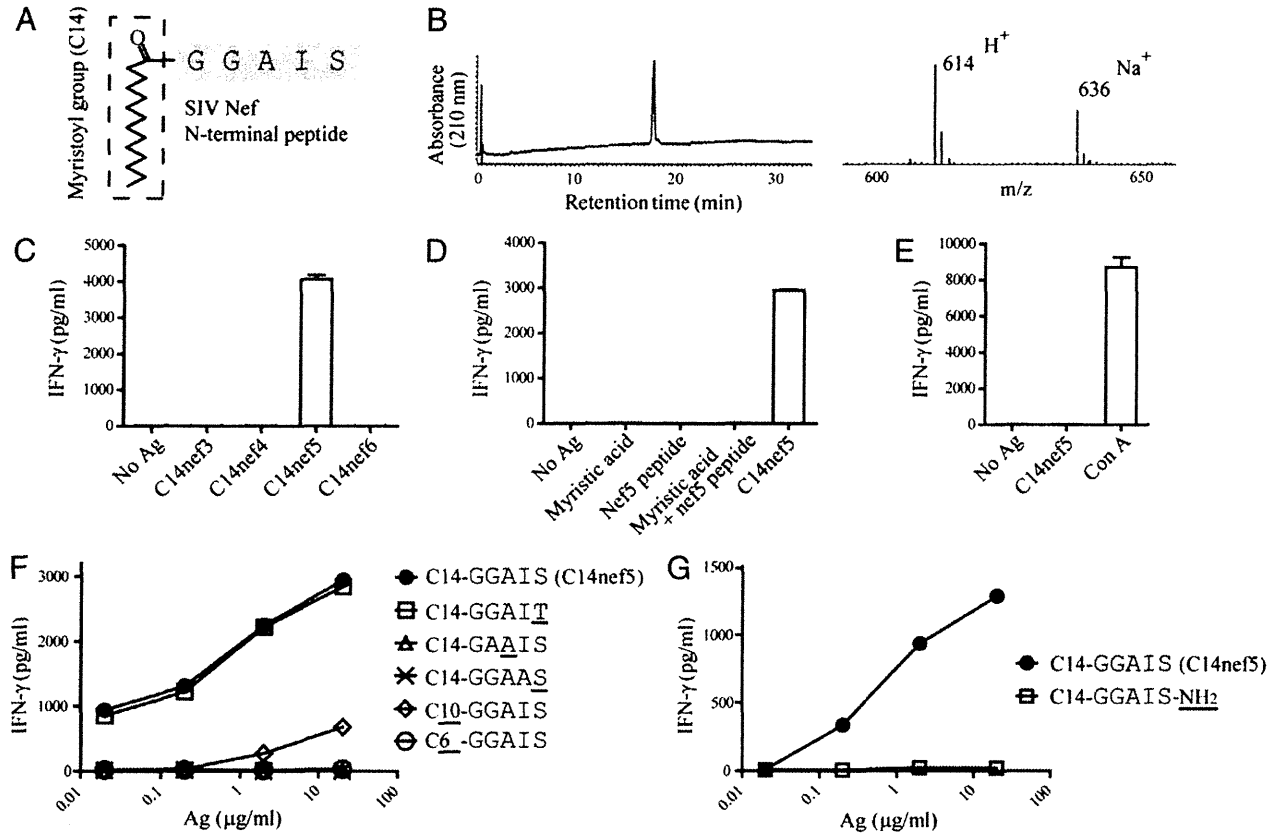

FIGURE 1. The response of 2N5.1 to N-myristoylated Nef peptides. *A*, The N-terminal 5-mer peptide derived from the mac239 strain of SIV was synthesized, and a myristic acid was conjugated with the glycine residue to generate the C14nef5 lipopeptide. *B*, The synthetic compound was analyzed by liquid chromatography mass spectrometry. A single peak was observed at a retention time of 19 min (*left panel*), and the ions with a mass-to-charge ratio of 614.4 and 636.4 were detected that corresponded to the proton and sodium adducts of C14nef5 with the exact mass of 613.41 (*right panel*). *C* and *D*, The 2N5.1 T cells (5×10^4/well) were stimulated with the indicated Ags (5 μg/ml) in the presence of irradiated autologous PBMCs (3×10^5), and the amount of IFN-γ released into the culture media was measured. Assays were performed in triplicate samples, and the mean values with the SD are shown. *E*, PBMCs (3×10^5/well) were incubated with medium, C14nef5, or Con A (5 μg/ml), and the amount of secreted IFN-γ was measured. *F* and *G*, The 2N5.1 T cells were stimulated with either C14nef5 or mutant Ags, and the amount of IFN-γ released into the media was measured.

CA) supplemented with 10% heat-inactivated FCS (Hyclone, Logan, UT), 2-ME (Invitrogen), penicillin, and streptomycin.

T cell assays

T cells (5×10^4/well) were incubated with the indicated concentrations of Ags in the presence of irradiated autologous or allogeneic PBMCs (3×10^5/well) using 96-well, flat-bottom microtiter plates. After 24 h, aliquots of the culture supernatants were collected, and the amount of IFN-γ released into the media was measured using a human/monkey IFN-γ ELISA kit (Mabtech, Nacka Strand, Sweden).

Flow cytometry

The surface expression of T cell markers on the 2N5.1 T cells was analyzed by flow cytometry as described previously (5). For the cytotoxicity assay, the T cell line was labeled by incubating the cells with 500 nM CFSE (Invitrogen) for 10 min at 37°C. Allogeneic PBMCs (3×10^5/well) were cultured in the presence or absence of the CSFE-labeled 2N5.1 T cells (1×10^5/well) and/or the C14nef5 Ag (5 μg/ml). After 4.5 h culture, the whole cells were harvested and stained with PE-labeled mouse mAbs to CD3 (SP34-2), CD14 (M5E2), or CD20 (2H7) for 30 min on ice. The labeled cells were washed, and propidium iodide (PI; BD Biosciences) was added to gate out dead cells. The cell samples were analyzed by flow cytometry using a BD FACSCanto II flow cytometer (BD Biosciences). Data were collected at a constant flow rate (120 μl/min).

Animals and viral infection

The rhesus macaques (*Macaca mulatta*) used in this study were treated humanely in accordance with the institutional regulations, and experimental protocols were approved by the Committee for Experimental Use of Nonhuman Primates at the Institute for Virus Research, Kyoto University. Six healthy monkeys were inoculated with SIVmac239 (6) i.v. at a dose of 2000 50% tissue culture-infective dose, and the titer of plasma viral RNA was determined by quantitative RT-PCR as described previously (7).

IFN-γ ELISPOT assays

ELISPOT plates (Millipore, Billerica, MA) were coated with the GZ-4 anti–IFN-γ Ab (10 μg/ml) at 4°C overnight. After blocking with RPMI 1640

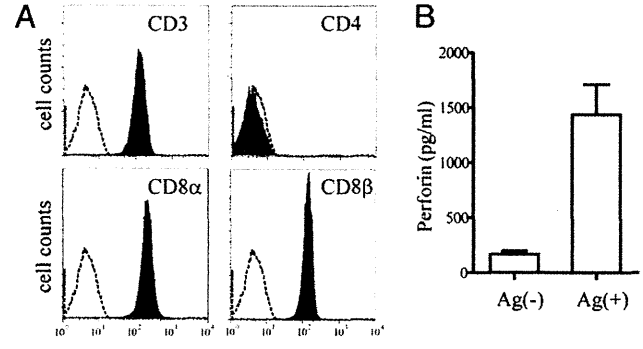

FIGURE 2. Expression of CD8 molecules and perforin by the 2N5.1 T cells. *A*, The 2N5.1 T cells were tested for their expression of T cell markers by flow cytometry. A dotted line in each panel indicates a histogram with a negative control Ab. *B*, The 2N5.1 T cells (5×10^4/well) were cultured with autologous PBMCs (3×10^5/well) in either the presence or the absence of the C14nef5 Ag (5 μg/ml), and the amount of perforin released into the culture media was measured.

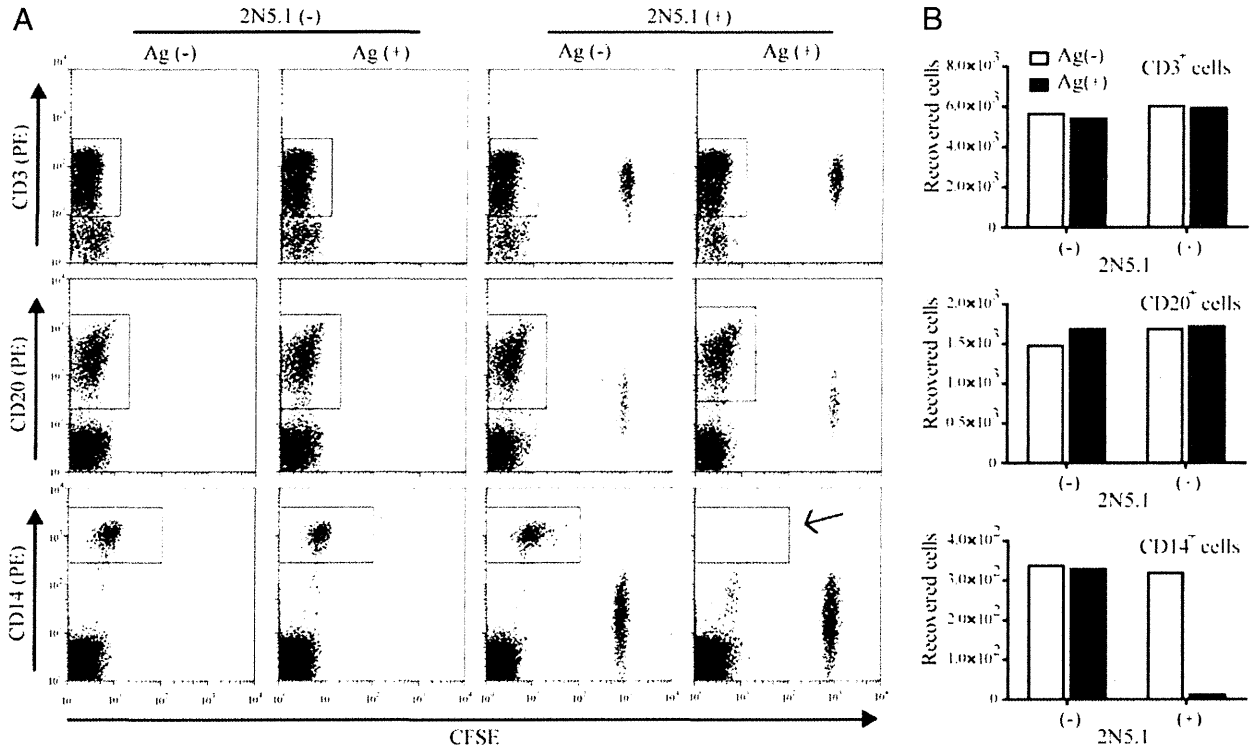

FIGURE 3. *Elimination of C14nef5-pulsed monocytes by the 2N5.1 T cells. A,* Allogeneic PBMCs (3×10^5/well) were incubated in either the presence or the absence of the CFSE-labeled 2N5.1 T cells (1×10^5/well) and the C14nef5 Ag for 4.5 h. The cells were then stained with PE-labeled Abs to CD3, CD20, or CD14, and PI-unstained live cells were analyzed by flow cytometry. PBMC-derived T cells, B cells, and monocytes that survived were boxed in the *top, middle,* and *bottom panels,* respectively. Note that monocytes were specifically eliminated when PBMCs were incubated in the presence of the 2N5.1 T cells and the C14nef5 Ag (indicated with an arrow). *B,* The absolute number of recovered $CD3^+$ (*top panel*), $CD20^+$ (*middle panel*), and $CD14^+$ cells (*bottom panel*) during 1 min of flow cytometric analysis is shown.

supplemented with 10% FCS, monkey PBMCs (2.5×10^5/well) were incubated with Ags (5 μg/ml) for 20 h. The plates were then washed with PBS, and spots representing IFN-γ–secreting cells were detected by sequential incubation with the biotinylated 7-B6-1 anti–IFN-γ Ab (1 μg/ml) for 2 h at room temperature, followed by HRP-conjugated streptavidin for 1 h and tetramethylbenzidine for 10 min (all from Mabtech). Samples were analyzed in duplicate, and the mean numbers of the spots were calculated.

Results and Discussion

To address whether T cells could specifically recognize N-myristoylated Nef peptides, PBMCs obtained from rhesus macaque (*M. mulatta*) monkeys were stimulated repeatedly in an in vitro culture with an array of synthetic N-myristoylated peptides derived from the SIV Nef protein. As a result of this attempt, a T cell line, termed 2N5.1, was established that proliferated in response to the N-myristoylated Nef 5-mer peptide (C14nef5) (Fig. 1*A*, 1*B*). The cells produced IFN-γ in response to C14nef5, but not N-myristoylated 3-mer (C14nef3), 4-mer (C14nef4), and 6-mer (C14nef6) of the Nef peptide (Fig. 1*C*). Furthermore, the 2N5.1 T cell activation was not observed when myristic acid and the 5-mer peptide were added as a free form (Fig. 1*D*), suggesting that

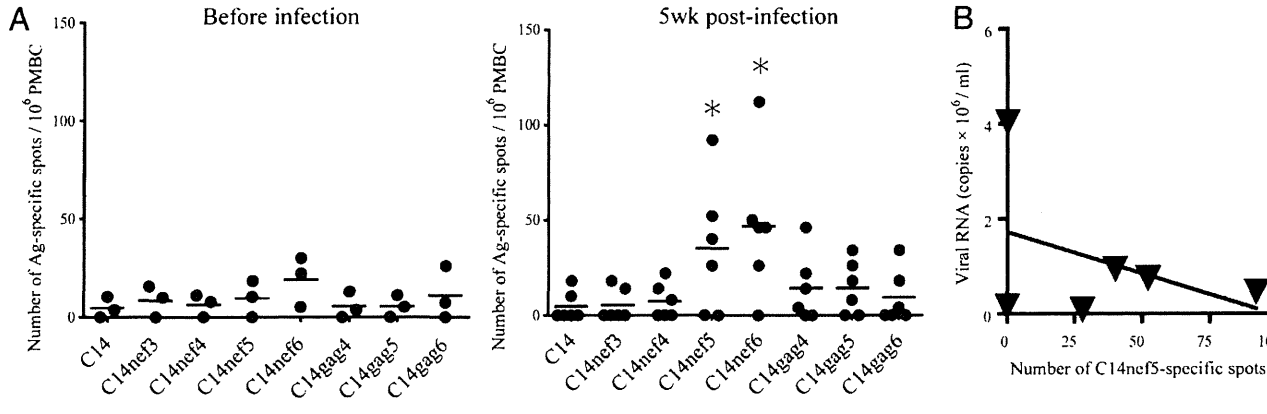

FIGURE 4. T cell responses to myristoylated Nef peptides in SIV-infected monkeys. *A,* Six healthy rhesus monkeys were inoculated with SIVmac239 i.v. at a dose of 2000 50% tissue culture-infective dose. Before ($n = 3$, *left panel*) and 5 wk postinfection ($n = 6$, *right panel*), PBMCs were obtained and stimulated with the indicated Ags, followed by detection of IFN-γ–producing cells in ELISPOT assays. The numbers of Ag-specific spots in individual subjects are shown with dots, and the mean values are indicated with bars. *B,* The numbers of C14nef5-specific spots and the virus load at 7 wk postinfection are plotted for each subject. The linear line was drawn on the basis of the linear least-square method, and the value of correlation coefficient was −0.41 ($n = 6$). *$p < 0.05$.

the T cells specifically recognized the 5-mer peptide that was conjugated covalently with myristic acid. Freshly isolated PBMCs responded to Con A, but not C14nef5 (Fig. 1*E*), ruling out the possibility that C14nef5 functioned as a nonspecific T cell mitogen.

An additional series of mutational analyses revealed a fine specificity for the lipopeptide recognition by the T cells. The 5-mer Nef peptide conjugated with a shorter (C_{10}) saturated fatty acid (C10-GGAIS) showed reduced T cell stimulation activity compared with C14nef5, and no T cell response was detected for C6-GGAIS (Fig. 1*F*), further confirming that the peptide modification with a fatty acid of the C_{14} chain length (myristic acid) was essential for activation of the 2N5.1 cells. The N-terminal amino acid sequence (GGAIS) of the Nef protein matches with a typical N-myristoylation motif, Gly-X-X-X-(Ser/Thr), in which X is any amino acid (3). Whereas the serine-to-threonine substitution (C14-GGAIT) did not affect the antigenic activity, alanine substitution for either the second glycine residue (C14-GAAIS) or the isoleucine residue (C14-GGAAS) located between the conserved flanking amino acid residues totally abrogated the activity (Fig. 1*F*). Furthermore, addition of an amide linkage to the carboxyl group of the C-terminal serine residue (C14-GGAIS-NH_2) resulted in total loss of the antigenic activity (Fig. 1*G*). These results indicated that the amino acid sequence of the C14nef5 lipopeptide constituted a T cell epitope that was short in length but still stringent in terms of amino acid selection compared with that recognized by classical MHC-restricted, peptide-specific T cells.

As expected from the highly specific recognition of peptide sequences, the 2N5.1 T cell line expressed clonotypic TCR α- and β-chains with random N-additions (data not shown). The 2N5.1 T cells were found to be CD4 negative and positive for CD8α- and CD8β-chains (Fig. 2*A*). Upon antigenic stimulation, the cells could secrete perforin (Fig. 2*B*), suggesting that the 2N5.1 T cells were both phenotypically and functionally defined as CTLs. To directly assess the Ag-dependent cytolytic activity exerted by the T cells, freshly isolated PBMCs were used as target cells for CFSE-labeled 2N5.1 T cells in an in vitro culture in either the presence or absence of the C14nef5 Ag. After 4.5 h, all of the cells were harvested and labeled with PE-conjugated Abs to CD3, CD20, or CD14. Whereas PI-stained dead cells were gated out, PBMC-derived T cells ($CD3^+$, $CSFE^-$ cells), B cells ($CD20^+$ cells), and monocytes ($CD14^+$ cells) as well as the 2N5.1 effector cells ($CSFE^+$ cells) were monitored by flow cytometry (Fig. 3*A*). The cell populations representing the recovered target T cells (Fig. 3*A*, boxed in the *top panels*) and B cells (Fig. 3*A*, boxed in the *middle panels*) were unchanged throughout the panels, and the absolute numbers of the cells were virtually the same regardless of the presence or absence of the T cells and the C14nef5 Ag (Fig. 3*B*, *top* and *middle panels*). In sharp contrast, the number of recovered $CD14^+$ monocytes was markedly reduced after culture in the presence of both the 2N5.1 T cells and the C14nef5 Ag (indicated with an arrow in Fig. 3*A* and shown in the *bottom panel* of Fig. 3*B*), demonstrating Ag-dependent killing of monocytes by the T cells. Consistent with this, peripheral blood monocytes purified by an MACS-based procedure could fully stimulate the 2N5.1 cells to produce IFN-γ (data not shown). On the basis of these observations, we concluded that the C14nef5-specific T cells were CTLs with monocytes as a major target cell type.

The results described above underscored the capacity for T cells to recognize the myristoylated Nef peptide, but the relevance of this to SIV infection in vivo remained to be established. We therefore wished to determine if such T cell responses directed specifically against myristoylated viral peptides might be elicited in SIV-infected monkeys. PBMCs were obtained from monkeys before and 5 wk postinfection with the mac239 strain of SIV and tested for their reactivity to C14nef3, C14nef4, C14nef5, C14nef6, and three N-terminally myristoylated SIV Gag peptides, C14-GVRN (C14gag4), C14-GVRNS (C14gag5), and C14-GVRNSV (C14gag6), in IFN-γ ELISPOT assays. Whereas no Ag-specific T cell responses were detected before infection, the number of T cells that recognized C14nef5 was significantly increased after SIV infection (Fig. 4*A*), suggesting that the immune recognition of C14nef5 indeed occurred in response to SIV infection. In addition, a significant fraction of T cells recognized C14nef6 in infected monkeys, which had not been expected from the study with the 2N5.1 T cell line. Finally, the plasma viral load at 7 wk postinfection in each SIV-infected monkey appeared to correlate reciprocally with the number of C14nef5-specific T cells (Fig. 4*B*).

It has been a challenge over the past two decades to develop effective vaccines against human infection with HIV. Unfortunately, the development of HIV vaccines designed for activating classical peptide-specific, MHC class I-restricted CTLs has had only limited success so far (8). The vaccine potential of the myristoylated Nef peptides will be tested directly, but one can predict that this new class of lipopeptide vaccine candidates may have a couple of important advantages over classical protein/peptide vaccines. Introducing amino acid mutations in the target proteins is an efficient strategy that HIV has evolved to escape from CTL attack, but the short stretch of the N-terminal amino acid residues of the Nef protein that contains N-myristoylation signal is hard to mutate without affecting the function of the protein (4). Although our preliminary studies indicated that the lipopeptide presentation could occur independently of CD1 function, the C14nef5-specific 2N5.1 T cell response was elicited by using PBMCs from any donor rhesus macaque monkeys as APCs, suggesting that the response could potentially be mediated by nonpolymorphic elements shared in all of the individuals. HIV uses the host cellular machinery for N-myristoylation of the Nef protein to favor its replication in the host, but the current study indicates that this results in unintended expansion of the Ag repertoire recognized by the host immune system. Lipid modifications are critical for a significant number of pathogenic viral proteins to function, but the host immunity appears to have evolved the ability to sense this post-translational event.

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Disclosures

The authors have no financial conflicts of interest.

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Dominant induction of vaccine antigen-specific cytotoxic T lymphocyte responses after simian immunodeficiency virus challenge

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ABSTRACT

Cytotoxic T lymphocyte (CTL) responses are crucial for the control of human and simian immunodeficiency virus (HIV and SIV) replication. A promising AIDS vaccine strategy is to induce CTL memory resulting in more effective CTL responses post-viral exposure compared to those in natural HIV infections. We previously developed a CTL-inducing vaccine and showed SIV control in some vaccinated rhesus macaques. These vaccine-based SIV controllers elicited vaccine antigen-specific CTL responses dominantly in the acute phase post-challenge. Here, we examined CTL responses post-challenge in those vaccinated animals that failed to control SIV replication. Unvaccinated rhesus macaques possessing the major histocompatibility complex class I haplotype *90-088-Ij* dominantly elicited SIV non-Gag antigen-specific CTL responses after SIV challenge, while those induced with Gag-specific CTL memory by prophylactic vaccination failed to control SIV replication with dominant Gag-specific CTL responses in the acute phase, indicating dominant induction of vaccine antigen-specific CTL responses post-challenge even in non-controllers. Further analysis suggested that prophylactic vaccination results in dominant induction of vaccine antigen-specific CTL responses post-viral exposure but delays SIV non-vaccine antigen-specific CTL responses. These results imply a significant influence of prophylactic vaccination on CTL immunodominance post-viral exposure, providing insights into antigen design in development of a CTL-inducing AIDS vaccine.

1. Introduction

In human and simian immunodeficiency virus (HIV and SIV) infections, cytotoxic T lymphocyte (CTL) responses exert strong suppressive pressure on viral replication but fail to control viremia leading to AIDS progression [1–5]. A promising AIDS vaccine strategy is to induce CTL memory resulting in more effective CTL responses post-viral exposure compared to those in natural HIV infections. It is important to determine how prophylactic CTL memory induction affects CTL responses in the acute phase post-viral exposure.

We previously developed a prophylactic AIDS vaccine (referred to as DNA/SeV-Gag vaccine) consisting of DNA priming followed by boosting with a recombinant Sendai virus (SeV) vector expressing SIVmac239 Gag [6]. Evaluation of this vaccine's efficacy against a SIVmac239 challenge in Burmese rhesus macaques showed that some vaccinees contained SIV replication [7]. In particular, vaccination consistently resulted in SIV control in those animals possessing the major histocompatibility complex class I (MHC-I) haplotype *90-120-Ia* [8]; $Gag_{206-216}$ (IINEEAADWDL) and $Gag_{241-249}$ (SSVDEQIQW) epitope-specific CTL responses were shown to be responsible for this vaccine-based SIV control [9]. Furthermore, in a SIVmac239 challenge experiment of *90-120-Ia*-positive macaques that received a prophylactic DNA/SeV vaccine expressing the $Gag_{241-249}$ epitope fused with enhanced green fluorescent protein (EGFP), all the vaccinees controlled SIV replication [10]. This single epitope vaccination resulted in dominant $Gag_{241-249}$-specific CTL responses with delayed $Gag_{206-216}$-specific CTL induction after SIV challenge, whereas $Gag_{206-216}$-specific and

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$Gag_{241-249}$-specific CTL responses were detected equivalently in unvaccinated *90-120-Ia*-positive animals.

These previous results in vaccine-based SIV controllers indicate dominant induction of vaccine antigen-specific CTL responses post-challenge, implying that prophylactic vaccination inducing vaccine antigen-specific CTL memory may delay CTL responses specific for viral antigens other than vaccine antigens (referred to as non-vaccine antigens) post-viral exposure. In these SIV controllers, the reduction of viral loads could be involved in delay of SIV non-vaccine antigen-specific CTL responses. Then, in the present study, we examined the influence of prophylactic vaccination on immunodominance post-challenge in those vaccinees that failed to control SIV replication. Our results showed dominant induction of vaccine antigen-specific CTL responses post-challenge even in these SIV non-controllers.

2. Materials and methods

2.1. Animal experiments

The first set of experiment used samples in our previous experiments of six Burmese rhesus macaques (Macaca mulatta) possessing the MHC-I haplotype *90-088-Ij* (macaques R02-004, R02-001, and R03-015, previously reported [7,11]; R04-014, R06-022, and R04-011, unpublished). Three of them, R02-001, R04-011, and R03-015, received a prophylactic DNA/SeV-Gag vaccine [7]. The DNA used for the vaccination, CMV-SHIVdEN, was constructed from *env*-deleted and *nef*-deleted simian–human immunodeficiency virus $SHIV_{MD14YE}$ [12] molecular clone DNA (SIVGP1) and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx, SIVmac239-HIV chimeric Vpr, 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 (CIUs) of F-deleted replication-defective SeV-Gag [13,14]. All six *90-088-Ij*-positive animals including three unvaccinated and three vaccinated were challenged intravenously with 1000 50% tissue culture infective doses (TCID50) of SIVmac239 [15] approximately 3 months after the boost. At week 1 after SIV challenge, macaque R03-015 was inoculated with nonspecific immunoglobulin G as previously described [11].

In the second set of experiment, unvaccinated (R06-001) and vaccinated (R05-028) rhesus macaques possessing the MHC-I haplotype *90-120-Ib* were challenged intravenously with 1000 TCID50 of SIVmac239. The latter R05-028 were immunized intranasally with F-deleted SeV-Gag approximately 3 months before the challenge.

In the third, three rhesus macaques received FMSIV plus mCAT1-expressing DNA vaccination three times with intervals of 4 weeks. The FMSIV DNA was constructed by replacing *nef*-deleted $SHIV_{MD14YE}$ with Friend murine leukemia virus (FMLV) *env*, carrying the same SIVmac239-derived antigen-coding regions with SIVGP1, as described before [16]. Vaccination of macaques with FMSIV and a DNA expressing the FMLV receptor (mCAT1) [17] three times with intervals of a week was previously shown to induce mCAT1-dependent confined FMSIV replication resulting in efficient CTL induction while vaccination three times with intervals of 4 weeks in the present study resulted in marginal levels of responses (data not shown). These three DNA-vaccinated animals were challenged intravenously with 1000 TCID50 of SIVmac239 approximately 2 months after the last vaccination.

Some animal experiments were conducted in the Tsukuba Primate Research Center, National Institute of Biomedical Innovation, with the help of the Corporation for Production and Research of Laboratory Primates, in accordance with the guidelines for animal experiments at the National Institute of Infectious Diseases, and others were in Institute for Virus Research, Kyoto University in accordance with the institutional regulations.

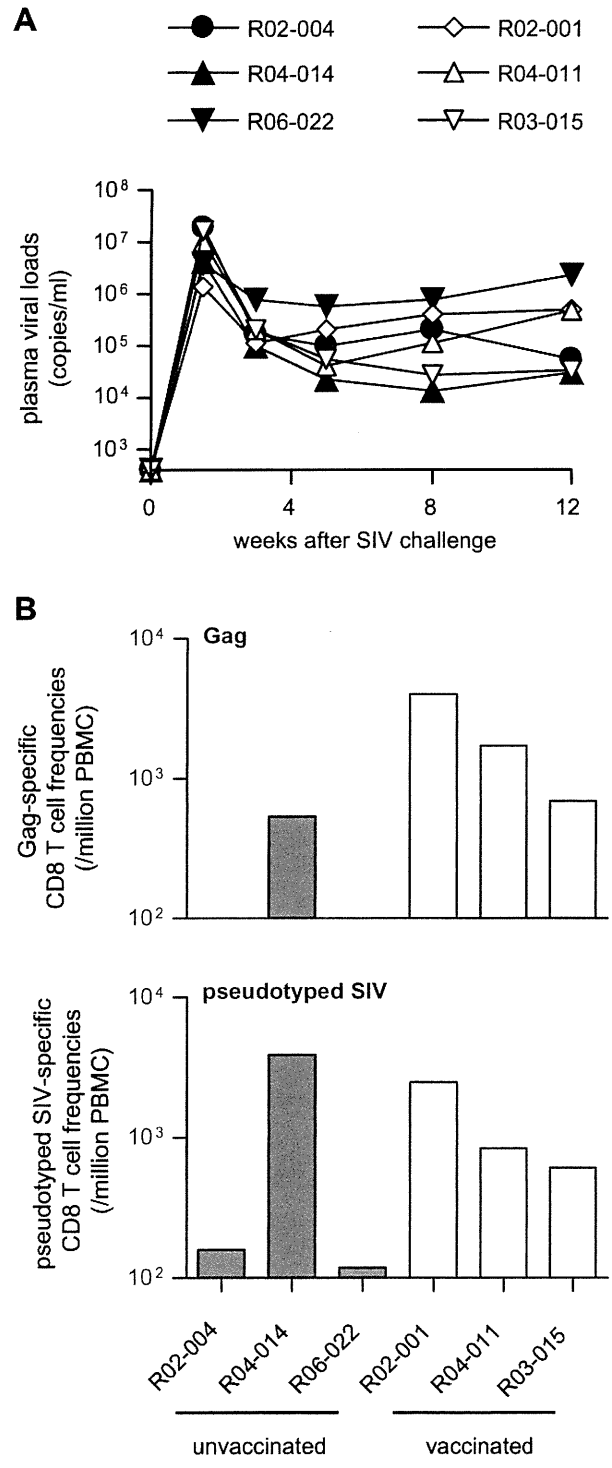

Fig. 1. CTL responses after SIVmac239 challenge in *90-088-Ij*-positive macaques. (A) Plasma viral loads after SIV challenge in unvaccinated (R02-004, R04-014, and R06-022) and DNA/SeV-Gag vaccinated animals (R02-001, R04-011, and R03-015). The viral loads (SIV gag RNA copies/ml) were determined as described previously [7]. (B) Vaccine antigen Gag-specific (upper panel) and pseudotyped SIV-specific $CD8^+$ T cell frequencies (lower panel) at week 2 after SIV challenge.

2.2. Analysis of virus-specific CTL responses

We measured virus-specific $CD8^+$ T-cell levels by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific

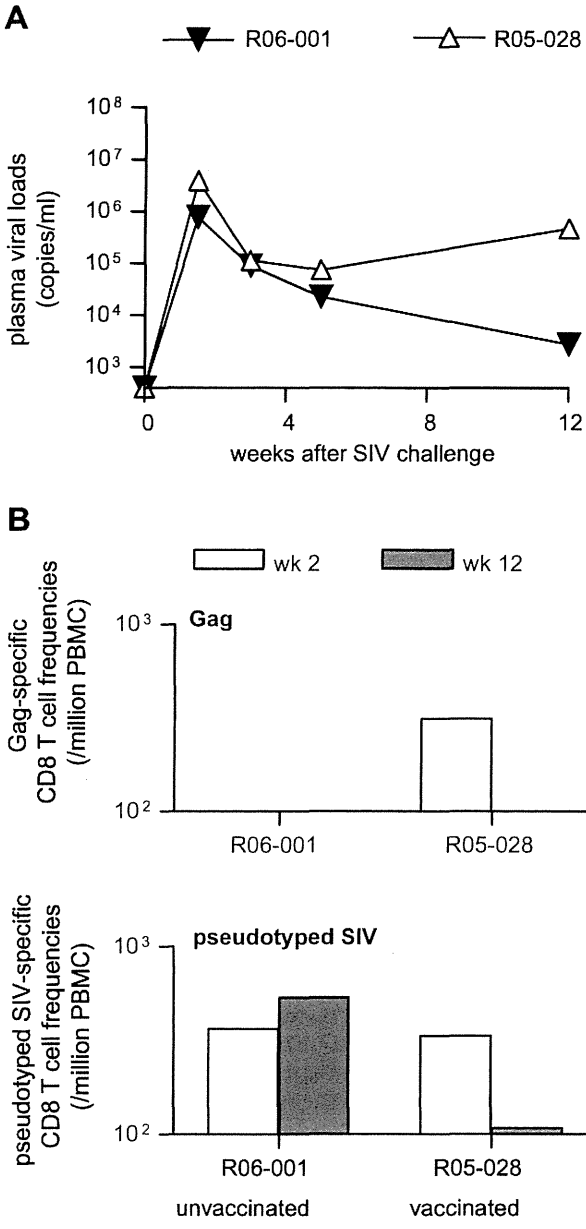

Fig. 2. CTL responses after SIVmac239 challenge in *90–120-Ib*-positive macaques. (A) Plasma viral loads after SIV challenge in unvaccinated R06-001 and SeV-Gag-vaccinated macaque R05-028. (B) Vaccine antigen Gag-specific (upper panel) and pseudotyped SIV-specific $CD8^+$ T cell frequencies (lower panel) at weeks 2 (white bars) and 12 (black bars) after SIV challenge.

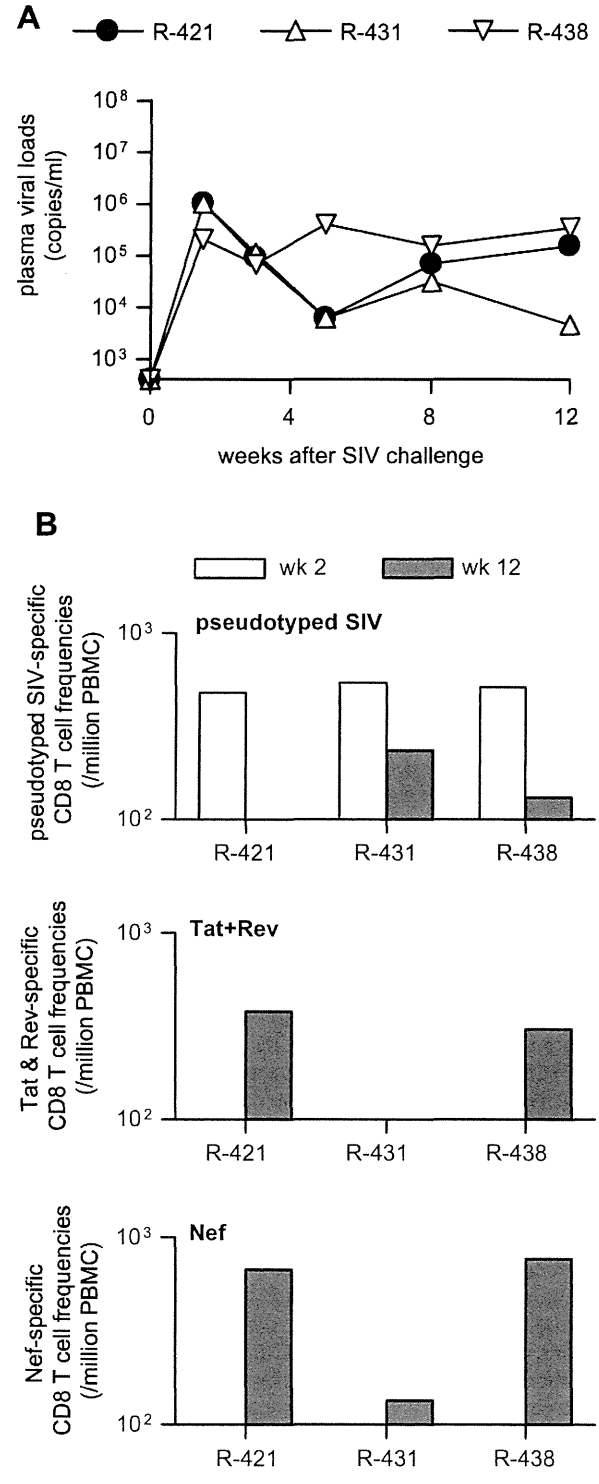

Fig. 3. CTL responses after SIVmac239 challenge in DNA-vaccinated macaques. The DNA used for the vaccination has the SIVmac239-derived region encoding Gag, Pol, Vif, and Vpx and is expected to induce pseudotyped SIV-specific CTL responses. (A) Plasma viral loads after SIV challenge in DNA vaccinated macaques R-421, R-431, and R-438. (B) Vaccine antigen (pseudotyped SIV)-specific (top panel), Tat-plus-Rev-specific (middle panel), and Nef-specific $CD8^+$ T cell frequencies (bottom panel) at weeks 2 (white bars) and 12 (black bars) after SIV challenge. In macaque R-438, CTL responses at week 5 instead of week 12 are shown.

stimulation as described previously [18,19]. Peripheral blood mononuclear cells (PBMCs) were cocultured with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines (B-LCLs) infected with a vaccinia virus vector expressing SIVmac239 Gag for Gag-specific stimulation or a vesicular stomatitis virus G protein (VSV-G)-pseudotyped SIV for pseudotyped SIV-specific stimulation. The pseudotyped SIV was obtained by cotransfection of COS-1 cells with a VSV-G-expression plasmid and SIVGP1 DNA. Alternatively, PBMCs were cocultured with B-LCLs pulsed with peptide pools using panels of overlapping peptides spanning the entire SIVmac239 Tat, Rev, and Nef amino acid sequences. Intracellular IFN-γ staining was performed with a CytofixCytoperm kit (Becton Dickinson, Tokyo, Japan) and fluorescein isothiocyanate-conjugated anti-human CD4, peridinin chlorophyll protein-conjugated anti-human CD8, allophycocyanin-conjugated anti-human CD3, and phycoerythrin-conjugated anti-human IFN-γ monoclonal antibodies (Becton Dickinson). Specific $CD8^+$ T-cell levels were calculated by subtracting nonspecific IFN-γ^+ $CD8^+$ T-cell frequencies from those after Gag-specific, pseudotyped

	vaccine antigen					non-vaccine antigen										
	Gag				Vif	Vpr	Tat					Rev		Nef		
	165	333	375	376	143	73	23	115	120	122	125	45	50	63	100	124
wk 5																
R- 421					++											
R- 431					+											
R- 438	++		+							++						
wk 12																
R- 421		++			++				+		+	+	+			++
R- 431					+		+			++						
R- 438	++			++		+		++						++	++	

Fig. 4. Viral mutations in DNA-vaccinated macaques. Plasma viral genome sequencing was performed as described previously [18] to determine mutations resulting in amino acid substitutions in SIV Gag, Pol, Vif, Vpx, Vpr, Tat, Rev, and Nef antigens (except for Env) at weeks 5 and 12 in DNA-vaccinated macaques. The amino acid positions showing mutant sequences dominantly (++) or equivalently with wild type (+) are shown. While we found a mutation leading to a lysine-to-arginine alteration at the 40th amino acid in Rev in all animals, this mutation is not shown because the wild-type sequence at this position in the SIVmac239 molecular clone is considered to be a suboptimal nucleotide that frequently reverts to an alternative sequence in vivo [18,23].

SIV-specific, or peptide-specific stimulation. Specific $CD8^+$ T-cell levels lower than 100 per million PBMCs were considered negative.

3. Results and discussion

In our previous SIVmac239 challenge experiments, the prophylactic DNA/SeV-Gag vaccination did not result in viral control in rhesus macaques possessing the MHC-I haplotype *90-088-Ij*. These vaccinated animals showed similar levels of plasma viral loads as those in unvaccinated *90-088-Ij*-positive animals after SIV challenge (Fig. 1A). Analysis of virus-specific $CD8^+$ T-cell responses using PBMCs at week 2 after challenge showed equivalent Gag-specific and pseudotyped SIV-specific (Gag-, Pol-, Vif-, and Vpx-specific) CTL responses in all three vaccinees (Fig. 1B). Pseudotyped SIV-specific CTL responses were also detected in all three unvaccinated animals, but Gag-specific CTL responses were undetectable in two out of the three; even the Gag-specific CTL responses detected in macaque R04-014 were much lower than pseudotyped SIV-specific CTL responses, indicating dominant induction of CTL responses specific for SIV antigens other than Gag (Fig. 1B). Thus, in the acute phase of SIV infection, SIV non-Gag antigen-specific CTL responses were dominantly induced in unvaccinated *90-088-Ij*-positive macaques, whereas vaccine antigen (Gag)-specific CTL responses were dominant in *90-088-Ij*-positive vaccinees.

We then analyzed another vaccinees that failed to control a SIVmac239 challenge; these macaques were vaccinated with SeV-Gag alone or DNA alone. First, we compared post-challenge CTL responses in unvaccinated and SeV-Gag-vaccinated macaques possessing the MHC-I haplotype *90-120-Ib*. Both macaques failed to control SIV replication after challenge (Fig. 2A). In the unvaccinated animal R06-001, Gag-specific CTL responses were undetectable but pseudotyped SIV-specific CTL responses were induced efficiently at weeks 2 and 12 (Fig. 2B). In contrast, Gag-specific CTL responses were induced efficiently at week 2 in the SeV-Gag-vaccinated animal R05-028 (Fig. 2B). At week 12, Gag-specific CTL responses became undetectable while pseudotyped SIV-specific CTL responses were still detectable in this animal. These results indicate that, in the acute phase after SIVmac239 challenge, the unvaccinated *90-120-Ib*-positive macaque dominantly elicited SIV non-Gag antigen-specific CTL responses whereas the SeV-Gag-vaccinated *90-120-Ib*-positive macaque dominantly induced vaccine antigen (Gag)-specific CTL responses.

Next, we analyzed post-challenge CTL responses in three DNA-vaccinated macaques. These animals failed to control SIVmac239 replication after challenge (Fig. 3A). The DNA used for the vaccination and the pseudotyped SIV genome both have the same SIVmac239-derived region encoding Gag, Pol, Vif, and Vpx, thus expected to induce pseudotyped SIV-specific CTL responses. Pseudotyped SIV-specific CTL responses, namely vaccine antigen-specific CTL responses, were induced efficiently at week 2 but diminished after that in all three animals (Fig. 3B). In contrast, Tat/Rev- and Nef-specific CTL responses were undetectable at week 2 but induced later (Fig. 3B). Again, vaccine antigen-specific CTL responses were dominantly induced in the acute phase after SIV challenge and non-vaccine antigen-specific CTL responses were elicited later.

All three animals showed viral genome mutations leading to amino acid substitutions in Gag or Vif at week 5 (Fig. 4). Further analysis indicated that viral mutations in vaccine antigen-coding regions appeared earlier than those in other regions. These results may reflect selective pressure on SIV by vaccine antigen-specific CTL responses dominantly induced in the acute phase, although it remains undetermined whether these mutations are CTL escape ones. Disappearance of vaccine antigen-specific CTL responses at week 12 may be explained by rapid selection of CTL escape mutations in vaccine antigen-coding regions. However, analysis using peptides found Gag-specific CTL responses in macaques R-421 and R-431 that had no gag mutations at week 5 (data not shown), suggesting involvement of immunodominance [20] in the disappearance of vaccine antigen-specific CTL responses at week 12.

In summary, the present study indicates that vaccine antigen-specific CTL responses are induced dominantly in the acute phase after viral exposure, with delayed induction of CTL responses specific for SIV non-vaccine antigens (SIV antigens other than vaccine antigens). While this delay previously-observed in vaccine-based SIV controllers [10] can be explained not only by immunodominance but also by reduction in viral loads, the delay in vaccinated non-controllers in the present study might reflect the immunodominance in CTL responses. Thus, in development of a prophylactic, CTL-inducing AIDS vaccine, it is important to select vaccine antigens leading to effective CTL responses post-viral

exposure [21,22]. These results imply a significant influence of prophylactic vaccination on the immunodominance pattern of CTL responses post-viral exposure, providing insights into antigen design in development of a CTL-inducing AIDS vaccine.

Acknowledgments

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Isolation of Potent Neutralizing Monoclonal Antibodies from an SIV-Infected Rhesus Macaque by Phage Display

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Abstract

The humoral immune response is a mechanism that potently suppresses or prevents viral infections. However, genetic diversity and resistance to antibody-mediated neutralization are serious obstacles in controlling HIV-1 infection. In this study, we isolated monoclonal antibodies from an SIV-infected macaque by using the phage display method to characterize antibodies in SIV infection. Variable regions of immunoglobulin genes were amplified by rhesus macaque-specific primers and inserted into the phagemid pComb3X, which produced the Fab fragment. Antibodies against SIV proteins were selected by biopanning using an SIV protein-coated 96-well plate. A total of 20 Fab clones obtained included 14 clones directed to gp41, four clones to gp120, and two clones to p27. The anti-gp120 Fab clones completely neutralized the homologous neutralization-sensitive SIVsmH635FC and the genetically divergent SIVmac316, and showed at least 50% inhibition against the neutralization-resistant strain, SIVsmE543-3. Competition ELISA revealed that these anti-gp120 Fab clones recognize the same epitope on gp120 including the V3 loop. Identification of antibodies with potent neutralizing activity will help to elucidate the mechanisms for inducing broadly neutralizing antibodies.

Introduction

THE HUMORAL IMMUNE RESPONSE is an important component of the adaptive immune response necessary to prevent viral infection and limit replication. Induction of these responses plays a key role in vaccine strategies against viral infection. Antibodies against human immunodeficiency virus type 1 (HIV-1), similar to those against other viruses, are capable of clearing HIV-1 virions.[1] Passive transfer of neutralizing antibodies (NAb) against HIV-1 to rhesus macaques provides sterilizing immunity against challenge with simian immunodeficiency virus (SIV)/HIV-1 chimeric viruses bearing the HIV-1 Env.[2–5] Although antibodies against a specific strain of HIV-1 can be highly potent, antibodies that neutralize a broad spectrum of HIV-1 strains are rarely induced in HIV-1-infected patients.[6,7] Since broad neutralization is critical for the development of a prophylactic vaccine against HIV-1, individuals who develop broadly NAbs are the subject of intense research.[8–12] In addition to the wide genetic diversity of HIV-1 limiting cross-neutralization, some strains of HIV-1 appear to be highly resistant to antibody-mediated neutralization. Many primary HIV-1 strains are resistant to neutralization, likely due to the structure of HIV-1 Env, which protects conserved regions from antibodies.[8,9,13] Consistent with the model of shielding of neutralizing epitopes, no broadly NAb has been obtained by immunization of mice or other animals with HIV-1 Env. Therefore, broadly neutralizing monoclonal antibodies (MAb) from HIV-1-infected patients are an essential but rare tool to understand the mechanism of neutralization against a broad spectrum of HIV-1 strains, including neutralization-resistant strains.

Macaques infected with SIV, which is genetically and biologically similar to HIV-1, are widely used as an animal model for HIV-1 infection.[14] The humoral immune response to SIV has been studied in the development of vaccine candidates and for exploration of antibodies that efficiently control viral infection. However, some SIV strains are known to be highly resistant to antibody neutralization, similar to the most resistant primary isolates of HIV-1.[15–17] The lack of MAbs that can neutralize these neutralization-resistant SIV strains is a major obstacle in the study of the mechanism of efficient neutralization using the SIV model.

Because conventional hybridoma technology is problematic in nonhuman primates, MAbs have been generated from rhesus macaques by the phage display method[18–21] or by transformation of B cells using rhesus Epstein–Barr virus-like virus.[22,23] In the present study, we used the phage display method to obtain MAbs against SIV antigens from an

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SIV-infected rhesus macaque with robust envelope-specific antibody responses. By panning with whole SIV antigen, we retrieved MAbs specific for SIV Env gp120, gp41, and Gag p27, and demonstrated the neutralization of a neutralization-resistant strain, SIVsmE543-3, by the gp120-specific MAbs.

Materials and Methods

Cells and viruses

PM1 cells[24] were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. TZM-bl,[25–28] GHOST(3) Hi-5,[29] and 293T[30] cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Lymphocytes from inguinal lymph nodes of SIVsmH635FC-infected rhesus macaque, H723,[31–33] were provided by Dr. Vanessa M. Hirsch. This animal died at 68 weeks postinfection with thrombus, bacterial endocarditis, and lymphadenopathy.[33] Peripheral blood samples were obtained from naive rhesus macaques, MM327, MM464, and MM449. These macaques were treated in accordance with the institutional regulations approved by the Committee for Experimental Use of Non-human Primates in the Institute for Virus Research, Kyoto University, Japan. Lymphocytes were isolated from blood samples by Ficoll gradient, and stored in liquid nitrogen until RNA preparation.

Infectious molecular clones SIVsmE543-3,[15] SIVsmH635FC,[32] SIVmac239,[34] SIVmac316,[35] and HIV-2_{GH123}[36] were transfected into 293T cells. After 2 days, the supernatants were filtered (0.45 μM) and stored at −80°C as virus stocks. The SIV antigen (Ag) used for panning was prepared by infection of PM1 cells with SIVsmE543-3. The culture supernatant was collected daily after 1 week postinfection and centrifuged at 12,000 rpm for 90 min at 4°C. After discarding the supernatant, the antigen pellet was resuspended in the remaining medium, treated with the same volume of PBS containing 1% Triton X-100, and stored at −80°C as SIV Ag.

Construction of Fab libraries

The Fab library was constructed using the pComb3X system according to instructions by Barbas *et al.*[37] The phagemid vector pComb3X was designed to express Fab or other proteins on the surface of phage with HA-tag and His-tag. Total RNA from lymphocytes was prepared using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). First-strand cDNAs were synthesized using oligo$(dT)_{20}$ primer and ReverTra Ace (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed by Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA). The first round of PCR was performed to amplify immunoglobulin (Ig) heavy chain variable region (VH), and light chain κ and λ variable regions (Vκ and Vλ) from cDNA sample using primers shown in Table 1 using the following condition: 94°C for 30 s, followed by 30 cycles of 94°C for 15 s, 55°C for 15 s, and 68°C for 60 s. Ig heavy chain γ constant domain 1 (CH1) and Ig light chain κ and λ constant domains (Cκ and Cλ) were similarly amplified using phagemid pComb3X with rhesus Ig genes as templates. These template phagemids were constructed by inserting PCR products that were amplified from rhesus macaque cDNA using primers, VH1a-*Xho*I (5′-CAG GTG CAG CTC GAG

TABLE 1. OLIGONUCLEOTIDE PRIMERS USED TO CONSTRUCT FAB LIBRARIES

VH 5′ sence primers	
RhFabVH17-F	GCTGCCCAACCAGCCATGGCCCAGGTSCAGCTGGTGCAGTCYGG
RhFabVH2-F	GCTGCCCAACCAGCCATGGCCCAGGTGACCTTGAAGGAGTCTGG
RhFabVH35-F	GCTGCCCAACCAGCCATGGCCGAGGTGCAGCTGGTGSAGTCTGG
RhFabVH46-F	GCTGCCCAACCAGCCATGGCCCAGGTGCAGCTGCAGGAGTCRGG
VH 3′ reverse primers	
RhFabVHJ1-B	CGATGGGCCCTTGGTGGAGGCTGAGGAGACGGTGACCAGGGCGCC
RhFabVHJ2-B	CGATGGGCCCTTGGTGGAGGCTGAGGAGATGGTGATTGGGGTGCC
RhFabVHJ36-B	CGATGGGCCCTTGGTGGAGGCTGAGGAGACGGTGACSMYGASCCC
RhFabVHJ45-B	CGATGGGCCCTTGGTGGAGGCTGAGGAGACGGTGACCAGGACTCC
Vκ 5′ sence primers	
RhSCK1-F	GGGCCCAGGCGGCCGAGCTCCAGATGWCCCAGTCTCC
RhSCK2-F	GGGCCCAGGCGGCCGAGCTCGTGATGAYCCAGACTCC
RhSCK23-F	GGGCCCAGGCGGCCGAGCTCGTRATGACKCAGTCTCC
RhSCK5-F	GGGCCCAGGCGGCCGAGCTCATACTCACACAGTCTGC
Vκ 3′ reverse primers	
RhCK5-B	GAAGACAGATGGTGCAGCCACAGC
Vλ 5′ sence primers	
RhSCLam131011	GGGCCCAGGCGGCCGAGCTCGDGCTGACWCAGCCACCCTC
RhSCLam2	GGGCCCAGGCGGCCGAGCTCGCCCYGACTCAGYCTCCCTCTGT
RhSCLam15	GGGCCCAGGCGGCCGAGCTCGTGCTGACSCAGCCKCCYTC
RhSCLam3a	GGGCCCAGGCGGCCGAGCTCGAGCTGACTCAGGAGCCTGCATTGTC
RhSCLam4	GGGCCCAGGCGGCCGAGCTCGTGCTGACTCAGTCGCCYTC
RhSCLam59	GGGCCCAGGCGGCCGAGCTCGTGCTGACTCAGCCRDCCTC
RhSCLam6	GGGCCCAGGCGGCCGAGCTCGTGTTCACTCAGCCCCATTC
RhSCLam78	GGGCCCAGGCGGCCGAGCTCGTRGTGACYCAGGAGCCMTC
Vλ 3′ reverse primers	
HCL5-B	CGAGGGGGCAGCCTTGGGCTGACC

CAG TCT GGG-3′) and CH-*Spe*I (5′-AGG TTT ACT AGT ACC ACC ACA TGT TTT TAT CTC-3′) for Ig heavy chain, VK1a-*Sac*I (5′-GAC ATC GAG CTC ACC CAG TCT CCA-3′) and CK-*Xba*I (5′-GCG CCG TCT AGA ATT AAC ACT CTC CCC TGT TGA AGC TCT TTG TGA CGG GCG AAC TCA G-3′) for Ig light chain κ, and VL1-*Sac*I (5′-GGG CCC AGG CGG CCG AGC TCG TGC TGA CGC AGC CTC CCT C-3′) and CL2-*Xba*I (5′-GCG CCG TCT AGA CCT ATG AAC ATT CTG CAG G-3′) for Ig light chain λ. Sequence data of Ig clones were submitted to GenBank under accession numbers FJ795797–FJ795868. The phagemids pComb3X-327w4LK10-327w4HC12 and pComb3X-327w4LL8 were used for this amplification as templates (GenBank accession numbers FJ795816, FJ795838, and FJ795863) using the following primers: HIgGCH1-F (5′-GCC TCC ACC AAG GGC CCA TCG GTC-3′), dpseq (5′-AGA AGC GTA GTC CGG AAC GTC-3′), RhKC-F (5′-CGA GCT GTG GCT GCA CCA TCT GTC-3′), HLC-F (5′-GGT CAG CCC AAG GCT GCC CCC-3′), and Lead-B (5′-GGC CAT GGC TGG TTG GGC AGC-3′). In second-round PCR, the heavy and light chains were constructed from the purified VH and CH1, Vκ and Cκ, and Vλ and Cλ fragments by overlap extension PCR using primers LeadVH (5′-GCT GCC CAA CCA GCC ATG GCC-3′), dpseq, RSC-F (5′-GAG GAG GAG GAG GAG GAG GCG GGG CCC AGG CGG CCG AGC TC-3′), and Lead-B: 94°C for 30 s, followed by 15 cycles of 94°C for 15 s, 55°C for 15 s, and 68°C for 90 s. Third-round PCR generated final Fab products by overlap extension PCR of heavy and light chains using primers RSC-F and dp-EX (5′-GAG GAG GAG GAG GAG GAG AGA AGC GTA GTC CGG AAC GTC-3′): 94°C for 30 s, followed by 10 cycles of 94°C for 15 s, 55°C for 15 s, and 68°C for 3 min. All PCR products were purified using the QIAquick Gel Extraction Kit or QIAEX II Gel Extraction Kit (QIAGEN) after loading them on an agarose gel. The final Fab fragments were ligated with pComb3X after digestion with *Sfi*I. The ligation mix was used for transformation of XL1-Blue (Stratagene, La Jolla, CA) by electroporation. Transformed cultures were incubated in SB medium with 50 μg/ml carbenicillin, 10 μg/ml tetracycline, and 1.4 μg/ml kanamycin overnight at 37°C after adding VCSM13 helper phage (Stratagene). Library phage stock was obtained from the culture medium by PEG 8000/NaCl precipitation. Library size was determined by colony-forming units (CFU) after infection of XL1-Blue with a diluted phage sample.

Biopanning using SIV Ag

Panning was performed using SIV Ag. Briefly, a MaxiSoap 96-well plate (Thermo Fisher Scientific, Waltham, MA) was coated with 50 μl/well of SIV Ag, which was 5-fold diluted with phosphate-buffered saline (PBS), for 1 h at 37°C. The 5-fold dilution was used because the signal by enzyme-linked immunosorbent assay (ELISA) was the strongest at this dilution. Wells were washed twice with PBS containing 0.05% Tween 20 (PBS-T) and were blocked with 5% skim milk (Wako Pure Chemical Industries, Osaka, Japan) in PBS (MPBS) for 1 h at 37°C. After discarding the blocking solution, 50 μl of phage library was added to each well, and the plate was incubated for 2 h at 37°C. After washing five times with PBS-T, bound phage were eluted with 50 μl 100 mM glycine (pH 2.2), and amplified for the next round of panning. A total of seven rounds of panning was performed.

To select Fab clones to SIV, phagemid DNA was transformed into TOP10F′ cells (Invitrogen). Bacterial colonies were cultured for 5 h at 37°C and Fab production was induced by adding isopropyl-ß-D-thiogalactopyranoside (IPTG, Wako Pure Chemical Industries) at a final concentration of 2 mM and shaking overnight at 37°C.

Large-scale Fab production and purification

A single colony was selected and cultured in 10 ml SB with 50 μg/ml carbenicillin at 30°C overnight. The bacterial culture was added to 200 ml to 1 liter SB medium with 20 mM $MgCl_2$ and 50 μg/ml carbenicillin and cultured for 8 h at 37°C. Fab production was induced by culturing overnight after adding IPTG at 1 mM. The bacterial pellet was resuspended in BugBuster Master Mix (Novagen, Madison, WI), and the soluble fraction was extracted according to the manufacturer's instruction. The clarified extract was loaded onto His GraviTrap (GE Healthcare, Buckinghamshire, UK) to purify histidine-tagged Fab. Purified Fab was concentrated and buffer exchanged to PBS by Vivaspin 6, 10 kDa MWCO (GE Healthcare).

ELISA assay to detect anti-SIV Fab

ELISA was performed to detect anti-SIV Fab. A MaxiSoap 96-well plate was coated with 50 μl/well of the 10-fold diluted SIV Ag for 1 h at 37°C. Wells were washed three times with PBS-T and blocked with MPBS for 1 h at 37°C or overnight at 4°C. Samples were added to each well at 50 μl/well, together with 50 μl of MPBS, and the plate was incubated for 1 h at 37°C. After washing three times with PBS-T, 100 μl of anti-HA-peroxidase (1:1000 dilution; 3F10, Roche Molecular Biochemicals, Mannheim, Germany) was added to each well, and the plate was incubated for 1 h at 37°C. After washing three times with PBS-T, 100 μl/well ABTS solution (Roche) was added, and the plate was incubated for 30 min at 37°C. The optical density (OD) at 405 nm was measured with reference OD_{490} using microplate reader (Model 550, Bio-Rad, Hercules, CA). The dilution of SIV Ag was determined from the result of ELISA using serially diluted SIV Ag. A sufficient signal was confirmed at the 40-fold dilution, and the 10-fold dilution was used for assays.

Con A SIV ELISA was performed to efficiently detect Fabs against gp120.[38] The plate was coated with PBS containing 50 ng/ml Con A (Sigma, St. Louis, MO) for 1 h at 37°C before adding SIV Ag to immobilize gp120, and processed similarly with SIV ELISA.

Competition ELISA

To determine the epitope specificity of Fab clones against gp120, a competition assay was performed using Con A ELISA. Ag-coated wells were incubated with 50 μl MPBS and 25 μl of serial dilutions of competitor, IgG-B404, for 1 h at 37°C. Subsaturating concentrations of Fab clones or murine MAbs, KK8, KK17, KK42, KK45, KK46, KK65, KK68,[39,40] and VM.18S, were added to each well at 25 μl/well, and the plate was incubated for 1 h at 37°C. After washing three times with PBS-T, 100 μl of anti-HA-peroxidase (1:1000) for Fab clones or antimurine IgG peroxidase (1:2000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) for murine MAbs was added to each well, and the plate was processed similarly with Con A ELISA.

Nucleic acid sequence analysis

For analysis of the Ig variable region, phagemid clones were sequenced using primers ompseq and pelseq.[37] Identical clones and defective clones were not used for further study. Complementarity-determining region 3 (CDR3) and the closest V gene allele were determined by comparison with human Ig genes using IMGT/V-QUEST in the International Immunogenetics Database (IMGT, http://imgt.cines.fr/).[41] Sequence data of Ig clones obtained were submitted to GenBank under accession numbers HM044964–HM045003.

Analysis of neutralizing antibody titers

A neutralization assay for kinetic analysis of antibody response in H723 was performed using GHOST(3) Hi-5 cells as previously described.[31] The highest dilution of plasma that resulted in a reduction in the number of GFP^+ cells by more than 90% is shown as the neutralization titer.

For screening of Fab clones, neutralizing ability was measured as the reduction in SIV^+ cells after infection to TZM-bl cells with SIVsmH635FC. Briefly, 100 μl of 10- and 30-fold diluted crude Fab samples in duplicate was incubated with 50 μl of 200 50% tissue culture infectious dose ($TCID_{50}$) of virus in a 96-well plate. After incubation for 1 h at 37°C, 100 μl of 1×10^5 TZM-bl cells/ml containing 18.75 μg/ml DEAE-dextran was added to each well. After 3 days, cells were washed with PBS, and fixed with PBS containing 2% formalin and 0.2% glutaraldehyde for 10 min at room temperature (RT). After washing twice, cells were stained with 0.5 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) in PBS containing 5 mM potassium hexacyanoferrate trihydrate, 5 mM potassium hexacyanoferrate, and 2 mM $MgCl_2$ for more than 1 h at 37°C. Fab clones that showed the reduction of stained cells were selected.

For the neutralization assay using purified Fab and plasma samples, the neutralizing ability was measured as the reduction in luciferase activity after infection to TZM-bl cells with various SIV strains, as previously described.[42] Briefly, 100 μl of serially diluted Fab samples in duplicate was incubated with 50 μl of 200 $TCID_{50}$ of virus in a 96-well plate for 1 h at 37°C, following addition of 100 μl of 1×10^5 cells/ml TZM-bl cells containing 37.5 μg/ml DEAE. Infected cultures were incubated for 2 days, but cultures infected with SIVsmH635FC were incubated for 3 days. After incubation, cells were lysed with 30 μl of cell lysing buffer (Promega, Madison, WI) for 15 min at RT, and 10 μl of cell lysate was transferred to a 96-well black solid plate (OptiPlates-96F, Perkin-Elmer, Boston, MA) for measurements of luminescence using a GloMax 96 Microplate Luminometer (Promega) and the luciferase assay system (Promega). The 50% and 90% inhibitory concentrations (IC_{90} and IC_{50}) were defined as the Fab concentration that caused a 50% and 90% reduction in luciferase activity compared to virus control wells after subtraction of background, respectively.

Western blot assay

Reactivity of plasma antibodies to SIV proteins was assessed by immunoblotting. SIV Ag was diluted with twice the volume of sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 25% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue), boiled for 5 min, and separated by SDS-polyacrylamide gel electrophoresis on 10% gel. Proteins were transferred to a Hybond-P PDVF membrane (GE Healthcare). The membrane was blocked with MPBS containing 0.1% Tween 20 for 1 h at RT, washed four times with PBS-T, and air dried on a filter paper. SIV strips were made by cutting the membrane, and stored at 4°C. For immunodetection, the SIV strip was incubated overnight at RT with 2 ml plasma from H723 (1:5000 dilution with MPBS containing 0.1% Tween 20), washed twice with PBS-T, and incubated for 1 h at RT with 2 ml protein A/G, and alkaline phosphatase conjugated (1:10,000 dilution with 0.5% milk PBS containing 0.1% Tween 20; Thermo Fisher Scientific). After washing three times with PBS-T, 1 ml BCIP/NBT solution (KPL, Gaithersburg, MD) was added to develop color.

Reactivity of Fab clones to SIV was also assessed using SIV strips. SIV strips were incubated overnight at 4°C with 1.8 ml MPBS containing 0.1% Tween 20 and 200 μl crude bacterial supernatant or purified Fab, washed twice with PBS-T, and incubated for 1 h at RT with 2 ml anti-HA-peroxidase (1:500 dilution; Roche). After washing three times with PBS-T, 1 ml TMB solution (KPL) was added to develop color. Bands for viral proteins were identified using SIV-positive sera and Donkey anti-IgG (H + L)-peroxidase (1:20,000 dilution; 709-035-149, Jackson ImmunoResearch, West Grove, PA) and confirmed by commercial kit (SIV Blot; ZeptoMetrix Corp., Buffalo, NY).

Flow cytometric analysis

The ability of Fabs to bind virus-infected cells was analyzed by flow cytometric analysis. Briefly, PM1 cells were infected with SIVsmE543-3, SIVmac239, SIVmac316, or $HIV\text{-}2_{GH123}$ and cultures were kept for 3 weeks by adding PM1 cells. Infected and uninfected cells were washed with PBS and adjusted to 5×10^6 cells/ml. For cell surface staining, 50 μl cells in PBS containing 0.2% BSA were incubated with 10 μl of 20 ng/μl Fab for 40 min at RT. After washing with PBS containing 0.2% BSA, cells were incubated with 50 μl of anti-HA (1:200; Roche) for 20 min at RT, followed by incubation with 50 μl of anti-rat-FITC (1:500; Santa Cruz Biotechnology) for 20 min at RT. For both surface and intracellular staining, 50 μl of 5×10^6 cells/ml PM1 cells was fixed by IC Fixation Buffer (eBioscience, San Diego, CA) for 20 min at RT and washed with permeabilization buffer (eBioscience) twice before incubation with Fab. Cells were stained similarly to the surface staining process, but permeabilization buffer was used for washing and dilution of antibodies. The stained cells were analyzed by guava easyCyte 8HT (Millipore, Billerica, MA). Percentages of Fab^+ cells were determined by gating cells with high FITC intensity using unstained cells as a negative control. Data analysis was performed using FlowJo (TreeStar, San Carlos, CA).

Conversion of Fab B404 into a complete rhesus IgG

Rhesus macaque heavy chain Ig genes were amplified from H723 cDNA by RT-PCR using primers, SPH-F (5′-GAG CTA GCG CCG CCA CCA TGG ACT GGA CCT GGA-3′) and SPH-R (5′-CGA AGC TTG CAC CGG TGG CTG CTG CCA CCA AG-3′) for the leader region and HIgCH1-F (5′-GCC TCC ACC AAG GGC CCA TCG GTC-3′) and CH-R (5′-TTG TTT AAA CTA TCA TTT ACC CGG AGA CAC GGA GA-3′)

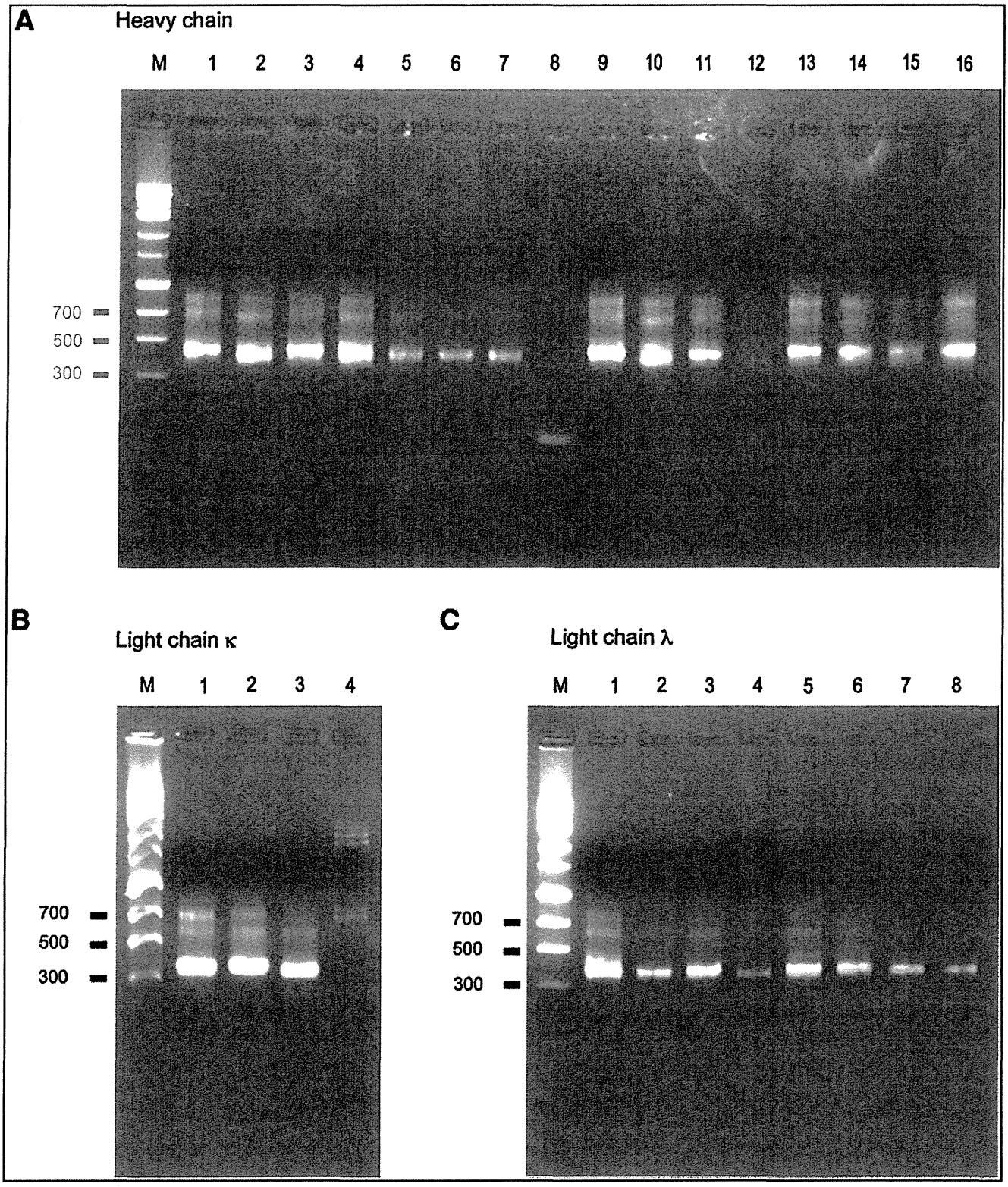

FIG. 1. Ig heavy chain and light chain κ and λ variable regions were successfully amplified by PCR using primers shown in Table 1. **(A)** The Ig heavy chain variable region was amplified using the following primer pairs: lane 1, RhFabVH17-F and RhFabVHJ1-B; 2, RhFabVH17-F and RhFabVHJ2-B; 3, RhFabVH17-F and RhFabVHJ36-B; 4, RhFabVH17-F and RhFabVHJ45-B; 5, RhFabVH2-F and RhFabVHJ1-B; 6, RhFabVH2-F and RhFabVHJ2-B; 7, RhFabVH2-F and RhFabVHJ36-B; 8, RhFabVH2-F and RhFabVHJ45-B; 9, RhFabVH35-F and RhFabVHJ1-B; 10, RhFabVH35-F and RhFabVHJ2-B; 11, RhFabVH35-F and RhFabVHJ36-B; 12, RhFabVH35-F and RhFabVHJ45-B; 13, RhFabVH46-F and RhFabVHJ1-B; 14, RhFabVH46-F and RhFabVHJ2-B; 15, RhFabVH46-F and RhFabVHJ36-B; and 16, RhFabVH46-F and RhFabVHJ45-B. **(B)** The Ig light chain κ variable region was amplified using RhCK5-B and the following primers: lane 1, RhSCK1-F; 2, RhSCK2-F; 3, RhSCK23-F; and 4, RhSCK5-F. (**C**) The Ig light chain λ variable region was amplified using HCL5-B and the following primers: lane 1, RhSCLam131011; 2, RhSCLam2; 3, RhSCLam15; 4, RhSCLam3a; 5, RhSCLam4; 6, RhSCLam59; 7, RhSCLam6; and 8, RhSCLam78. Template cDNA samples from four rhesus macaques were tested, and the representative result, which was obtained from macaque H723, is shown. M, molecular weight marker. The bands for 300 bp, 500 bp, and 700 bp are indicated on the left.

for the constant region. The leader and constant regions were inserted into pcDNA3.1(+) using restriction enzymes *Nhe*I and *Hin*dIII and *Apa*I and *Pme*I, respectively. The resultant plasmid, designated as pHCG, has an Ig heavy chain gene lacking the VH region. The VH region was amplified from Fab B404 using primers VH35-F (5′-GCC ACC GGT GCC CAC TCC GAG GTG CAG CTG GTG-3′) and VH-R (5′-CGA TGG GCC CTT GGT GGA G-3′) and inserted into pHCG after digestion with *Sgr*AI and *Apa*I. The B404 light chain gene, which was amplified using primers, Lam131011-F (5′-GDG CTG ACW CAG CCA CCC TC-3′) and CL2-*Xba*I, was combined with the light chain leader region, which was amplified using primers SPLa-F (5′-GAA AGC TTG CCG CCA CCA TGG CCT GGR CTC CWC-3′) and SPL131011-R (5′-GAG GGT GGC TGW GTC AGC HC-3′) by overlapping PCR using primers SPLa-F and CL2-*Xba*I. The PCR product containing the complete light chain gene was inserted into pcDNA3.1/Hyg(+) after digestion with *Hin*dIII and *Xba*I. The stable cell line expressing IgG-B404 was obtained by transfection of these plasmids into 293A cells and selection with 400 μg/ml G418 and 100 g/ml hygromycin. The culture supernatant was concentrated by Vivaflow 50, 30,000 MWCO (Sartorius Stedim Biotech, Goettingen, Germany), and IgG was purified by HiTrap rProtein A FF (GE Healthcare). Purified IgG was concentrated and buffer exchanged to PBS by Vivaspin 6, 10 kDa MWCO (GE Healthcare).

Results

Amplification of immunoglobulin V regions from macaque monkeys

Primers used to amplify variable regions of Ig genes were newly designed for rhesus macaques based on the primers for construction of a combinatorial library of human Ig genes,[37] germline sequences of rhesus Ig genes,[43–51] and the genome database of rhesus macaque[52] (Table 1). The Ig variable regions were amplified from lymphoid cells of SIVsmH635FC-infected macaque H723 and three uninfected macaques using these primers detailed in Table 1 (Fig. 1). Although most primers successfully amplified the Ig variable regions, some primers did not work well. For example, the reverse primer for the heavy chain, RhFabVHJ45-B, was not effective in amplifying a product in combination with the RhFabVH2-F and RhFabVH35-F primers (Fig. 1A, lanes 8 and 12). In addition, the sense primer for light chain κ, RhSCK5-F, was not effective (Fig. 1B, lane 4), even though this primer was designed based on a rhesus genome sequence that was similar to germline sequence of human IGKV5. Nevertheless, the

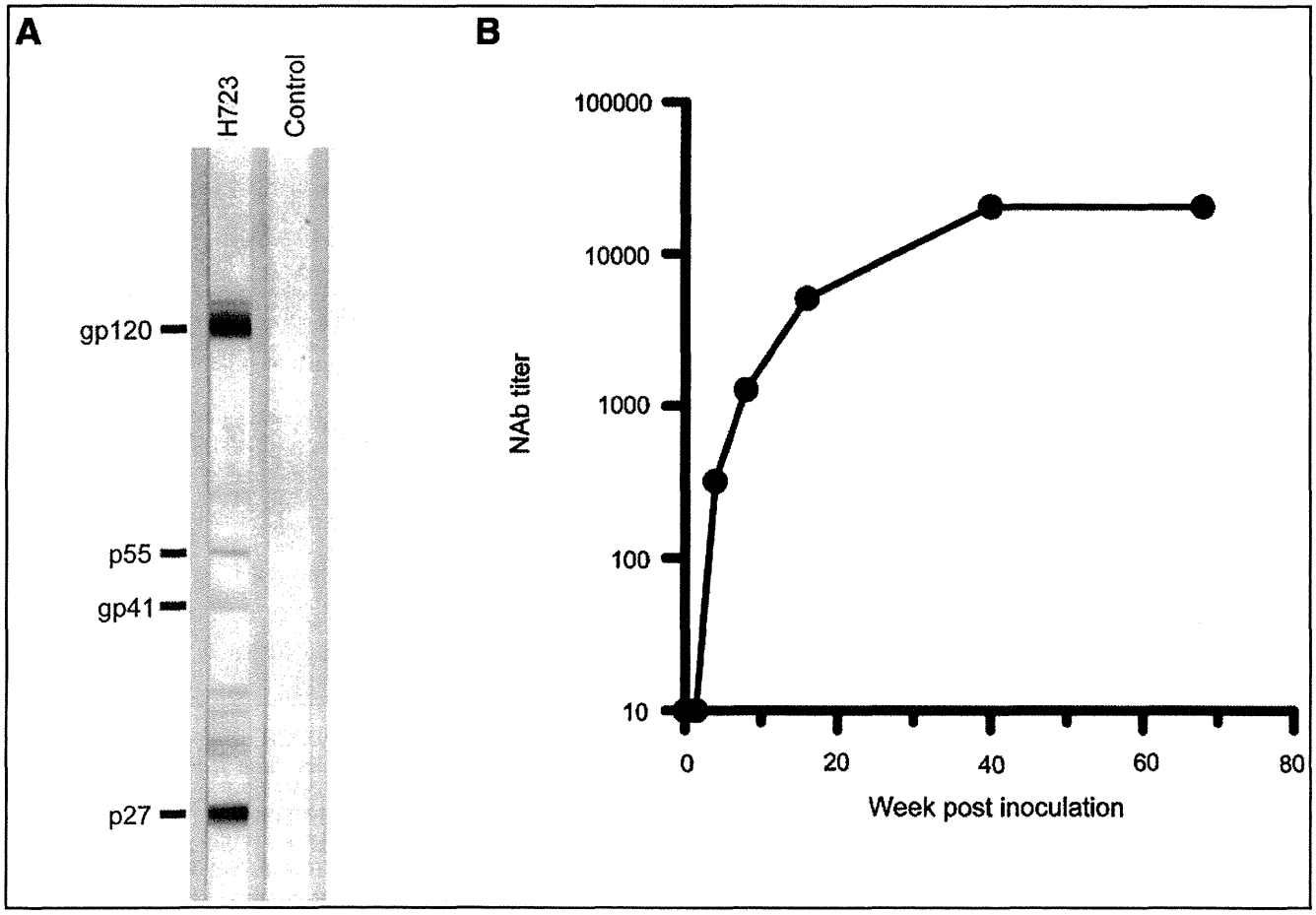

FIG. 2. Antibody response in SIVsmH635FC-infected macaque H723. **(A)** Western blot analysis showed that antibodies in plasma from H723 at death recognized Env gp120 and gp41 and Gag p55 and p27. **(B)** H723 maintained a high NAb titer during the course of infection. Neutralizing titer was determined by 90% inhibition of infection to GHOST(3) Hi-5 cells with SIVsmH635FC.