G418 (Gibco)/ml for 14 days. The colonies obtained were examined for the expression of TRIM5α by using the TaqMan PCR method according to the manufacturer's instructions (Applied Biosystems). Sequences of the probe and primers used to specifically detect each TRIM5α were as follows: MT4-TRIM5, forward primer (5′-AACCTGGAGAAGGAGGAGGAAGAC-3′), reverse primer (5′-CTGGGTCTGCTGCACCAT-3′), and probe (5′-FAM-TCAGTTTCAGAG TTCG-TAMRA-3′); HSC-F-TRIM5, forward primer (5′-AACCTGGAGAAG GAGAAAGAAGAC-3′), reverse primer (5′-CTGGGTCTGCTGCACCAT-3′), and probe (5′-FAM-TTCGTTTCAGACTTTG-TAMRA-3′); and CV1-TRIM5, forward primer (5′-AACCTGGAGAAGGAGGAAGAAGA-3′), reverse primer (5′-CTGGGTCTGCTGCACCAT-3′), and probe (5′-FAM-TCCGTTTCAGAC TTCG-TAMRA-3′). These primers amplify the coiled-coil region of TRIM5 genes. Plasmid DNA used for transfection served as a standard to determine the number of copies of TRIM5α transcripts. The parental TK-ts13 cells were totally negative for the primate TRIM5α expression. Clones expressing each TRIM5α at comparable levels (ca. 4 to 6 × 10^7 copies/μg of total RNA) were used for subsequent experiments.

To generate CV1-TRIM5α and HSC-F-TRIM5α cDNAs carrying a hemagglutinin (HA) tag (YPYDVPDYAA) at their C termini, cloned CV1-TRIM5α and HSC-F-TRIM5α cDNAs in pcDNA3.1 were used as templates for PCR amplification with a primer containing a nucleotide sequence corresponding to the HA tag fused with the C-terminal portion of TRIM5α. The C-terminal portion of TRIM5α fused with the HA tag (BamHI to NotI) and the N-terminal portion of TRIM5α (NotI to BamHI) were assembled on a pCEP4 vector (Invitrogen). To generate chimeric TRIM5α HSC-F+60tag, the 182-bp SphI-BamHI fragment of HSC-F-TRIM5α-tag was replaced with the corresponding 242-bp SphI-BamHI fragment of CV1-TRIM5α in the background of HSC-F-TRIM5α-tag. Conversely, the 242-bp SphI-BamHI fragment of CV1-TRIM5α was replaced with the 182-bp SphI-BamHI fragment of HSC-F-TRIM5α-tag in the background of CV1-TRIM5α-tag to generate CV1-60tag. PCR-based mutagenesis of HSC-F-TRIM5α-tag was performed to generate HSC-delete-tag, which possessed the 5′-proximal 84 bp of the SphI-BamHI fragment of CV1-TRIM5α in the background of HSC-F-TRIM5α-tag, and HSC-insert-tag, which possessed 3′-proximal 158 bp of the SphI-BamHI fragment of CV1-TRIM5α in the background of HSC-F-TRIM5α-tag. Similarly, CV1-delete-tag, which possessed the 3′-proximal 98 bp of the SphI-BamHI fragment of HSC-F-TRIM5α in the background of CV1-TRIM5α-tag, was generated by a PCR-based mutagenesis of CV1-TRIM5α-tag. The entire coding sequences of these TRIM5α-tags were then transferred to the NotI site of pSeV18+b(+). Recombinant Sendai viruses (SeVs) carrying various TRIM5α-tags were recovered according to a previously described method (32). The viruses passaged a second time in embryonated chicken eggs were used as stock for all experiments. The wild-type Z strain of SeV served as a control in all of the experiments.

To establish human cell lines which constitutively express primate TRIM5αs or their chimeras, human osteosarcoma C143 cells were transfected with pCEP4 containing cDNA of CV1-TRIM5α-tag, HSC-F-TRIM5α-tag, CV1-60tag, or HSC-F+60tag, and cells were cultured in the presence of 0.3 mg of hygromycin B (Gibco)/ml for 14 days.

Immunoprecipitation and Western blot analysis. When we performed Western blot analysis of cells expressing HA-tagged TRIM5α proteins, we consistently observed nonspecific binding of anti-HA antibody to a protein that comigrated with HSC-F-TRIM5α. Therefore, we analyzed the expression of each HA-tagged TRIM5α protein in the hygromycin B-resistant C143 cells or MT4 cells infected with recombinant SeVs by immunoprecipitation, followed by Western blot analysis as described previously (20) to reduce nonspecific background. Briefly, cell lysate was first adsorbed with protein A-agarose before the addition of anti-HA antibody to avoid nonspecific protein binding to protein A-agarose. TRIM5α proteins in the cell lysate were then precipitated with anti-HA high-affinity rat monoclonal antibody (Roche) by using a protein A-immunoprecipitation kit (Roche). Precipitated materials were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 4 to 12% NuPAGE Bis-Tris gel (Invitrogen). Proteins in the gel were then electronically transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore). Blots were blocked and probed with anti-HA antibody overnight at 4°C and then incubated with peroxidase-conjugated anti-rat immunoglobulin G (American Qualex) and developed by using the Immun-Star HRP chemiluminescence kit (Bio-Rad). Visualized image was recorded by LAS1000 (Fuji) and quantified by ImageGauge (Fuji). At least three independent experiments were performed, and the means and standard deviations (SD) for the data were calculated.

Viruses and HIV-1 lentivirus vector. VSV-G-pseudo typed HIV-1-NL43, SIVmac239, or HIV-2-GH123 was prepared by transfection of 293T cells with a combination of pMD.G (17, 18) and pNL432 (1), pBRmac239 (15), or pGH123 (30), respectively. HIV-1 vector expressing green fluorescence protein (GFP) was prepared as described previously (17, 18). Two days after transfection, culture supernatants of 293T cells were collected and assayed for reverse transcriptase activity using a reverse transcriptase colorimetric assay (Roche).

Viral infection. Assays for the HIV-1 vector expressing GFP were performed in 24-well plates containing 4 × 10^4 Tk-ts13-derived target cells. Serially diluted VSV-pseudotyped HIV-1 vectors encoding GFP were inoculated, and infected cells were enumerated by using a flow cytometer (FACScan; Becton Dickinson) 40 h after infection. For VSV-pseudotyped HIV-1, SIVmac239, and HIV-2 infection assays, we inoculated viruses containing 1 ng of reverse transcriptase into 4 × 10^4 C143 cells. For CD4-dependent infection assays, 2.5 × 10^5 MT4 cells were infected with SeV expressing CV1-TRIM5α-tag, HSC-F-TRIM5α-tag, or the parental Z strain of SeV at a MOI of 10 PFU per cell, followed by incubation at 37°C for 9 h. Cells were then superinfected with 30 ng of p24 of an X4 HIV-1 strain, NL43, or 30 ng of p27 of SIVmac239. The culture supernatants were collected periodically, and the level of p24 or p27 was measured by using a RETROtek antigen ELISA kit (ZeptoMetrix).

Data deposition. The sequences described here have been deposited in the GenBank database under accession numbers AB210050 to AB210052.

RESULTS

Variation in TRIM5α. We cloned TRIM5α cDNA from the human T-cell line MT4, cynomolgus monkey T-cell line HSC-F, and African green monkey kidney cell lines CV1 and Vero. The predicted amino acid sequences of TRIM5αs are compared in Fig. 1A and B.

Human TRIM5α from MT4 cells differed at an amino acid position 249 (G249D) from the previously published sequence (33) and was designated MT4-TRIM5α. The cynomolgus monkey TRIM5α from HSC-F (HSC-F-TRIM5α) was two amino acids shorter than the rhesus monkey TRIM5α (33) and two amino acids longer than the human TRIM5α. All 10 clones derived from Vero cells had the same sequence as the previously published one (36). On the other hand, we found at least two distinct TRIM5α sequences in CV1 cells. The two major TRIM5α sequences obtained from CV1 were designated CV1-TRIM5α-type1 and CV1-TRIM5α-type2, and five amino acids were found to differ between the two sequences (I259V, L337S, R351L, G359R, and G438S). Among 10 cDNA clones obtained from CV1, there were four type 1 clones and four type 2 clones. The remaining two clones were most likely chimeric artifacts. Two recently published sequences of TRIM5α from CV1 cells (AY593973 and AY625002) showed differences at three positions—L7V, I259V, and G438S—and have S, L, and R at positions 337, 351, and 359, respectively (14, 36), whereas both the type 1 and 2 clones had leucines at the seventh position. All sequences obtained from Vero and CV1 contained a 20-amino-acid duplication within the SPRY domain, which was not observed in human MT4 and cynomolgus monkey HSC-F (Fig. 1B).

The phylogenetic tree of various TRIM5α sequences showed that cynomolgus and rhesus monkey TRIM5αs are similar to each other, a finding consistent with the fact that these two monkeys belong to the genus *Macaca* (Fig. 1C).

African green monkey and cynomolgus monkey TRIM5α inhibit HIV-1 infection in nonprimate cells. We first sought to determine whether or not each TRIM5α can inhibit HIV-1 infection in the context of nonprimate cells because human and primate cells express endogenous TRIM5α that could complicate a functional analysis of TRIM5α-mediated restriction. The hamster cell line TK-ts13 was used, because it is very susceptible to a VSV-G-pseudotyped, HIV-1-based GFP-expressing lentivirus vector, HIV-1-GFP. Cell clones stably ex-

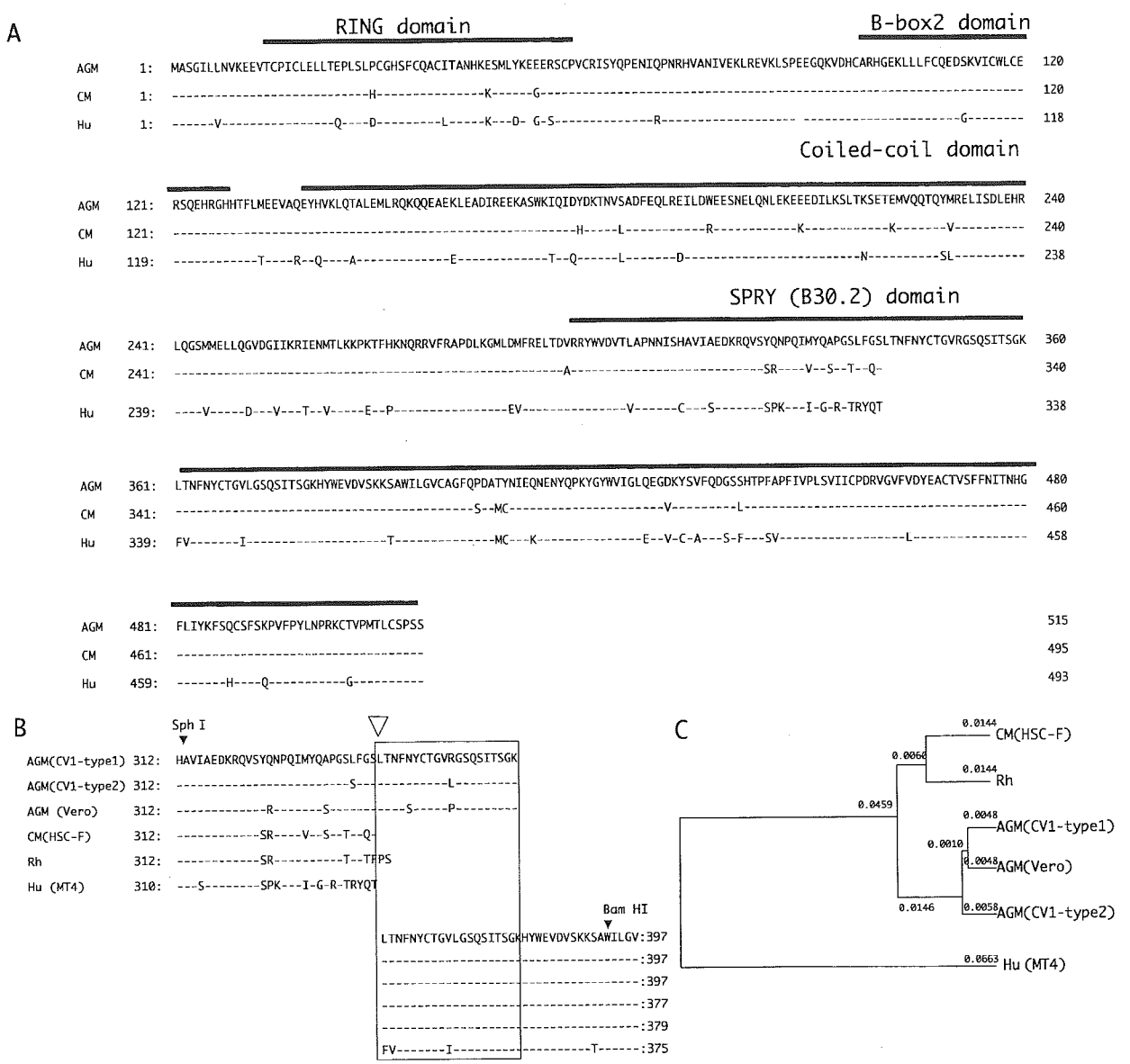

FIG. 1. (A) Alignment of amino acid sequences of African green monkey (AGM, CV1-TRIM5α-type1), cynomolgus monkey (CM, HSC-F-TRIM5α), and human TRIM5α (Hu, MT4-TRIM5α) predicted from the sequences of the cDNAs used in the present study with key domains indicated. (B) Alignment of amino acid sequences of the highly variable region within the SPRY (B30.2) domain of TRIM5α. The rhesus monkey TRIM5α (Rh) sequence published by Stremlau et al. (33) was added. A box indicates the 20-amino-acid duplication within African green monkey TRIM5α. An open arrowhead denotes the recombination point in chimeric TRIM5αs, HSC-delete-tag, HSC-insert-tag, and CV1-delete-tag (see Fig. 4A). (C) Phylogenetic tree of various TRIM5α sequences produced by the UPGMA (unweighted pair-group method with arithmetic averages) method.

pressing MT4-, CV1-, and HSC-F-TRIM5α were selected according to the method described in Materials and Methods. The levels of expression of TRIM5α were determined by using a real-time PCR, and cells expressing comparable amounts of TRIM5α (4×10^6 to 6×10^6 copies/μg of total RNA) were used for subsequent study. Restriction can be quantified by comparing the percentage of GFP-positive cells with or without TRIM5α.

As can be seen in Fig. 2A, MT4-TRIM5α had a very weak anti-HIV-1 effect (~1.2-fold), a finding consistent with a previous study (33). In contrast, restriction of HIV-1 was clearly evident over a wide range of initial MOIs in cells expressing HSC-F-TRIM5α, CV1-TRIM5α-type1, and CV1-TRIM5α-type2 (ca. 5- to 10-fold). There was no significant difference between CV1-TRIM5α-type1 and type2; therefore, we chose CV1-TRIM5α-type1 to be representative of CV1-TRIM5α in the subsequent experiments. We obtained the same results as described above when we used an HA-tagged version of TRIM5α (data not shown).

African green monkey and cynomolgus monkey TRIM5α inhibit CD4-dependent HIV-1 infection in human cells. To test the restriction properties of TRIM5α in CD4-dependent

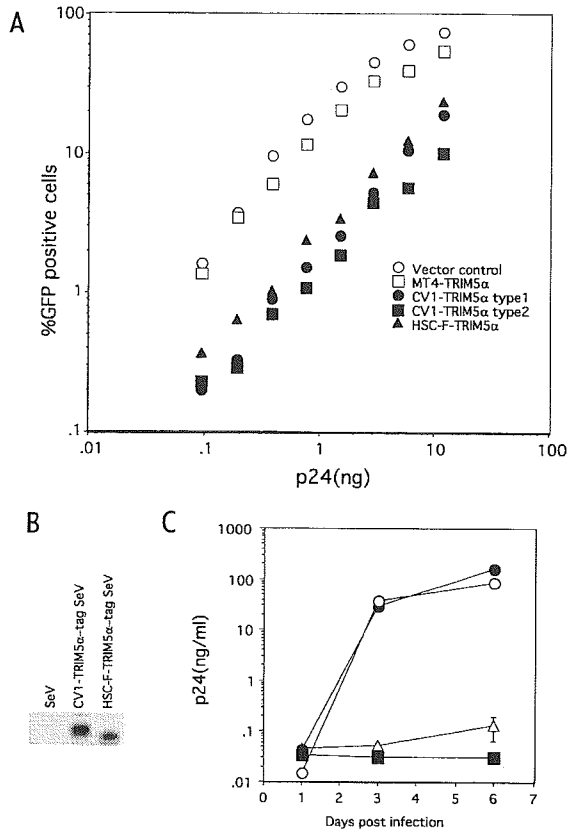

FIG. 2. (A) TK-ts13 cell clones expressing MT4-TRIM5α (□), CV1-TRIM5α-type1 (●), CV1-TRIM5α-type2 (■), HSC-F-TRIM5α (▲), or empty vector (○) were exposed to the indicated GFP-expressing HIV-1 vector. GFP-positive cells were counted with a flow cytometer. Data typical of at least three independent clones for each TRIM5α are shown. (B) Lysates of MT4 cells infected with recombinant SeV expressing CV1-TRIM5α-tag, HSC-F-TRIM5α-tag, or the parental Z strain were immunoprecipitated by anti-HA antibody. Resultant immunoprecipitates were visualized by Western blotting with an antibody to HA. A representative result of four independent experiments is shown. (C) MT4 cells were mock infected (○), or infected with SeV expressing CV1-TRIM5α-tag (■), HSC-F-TRIM5α-tag (△), or the parental Z strain (●). At 9 h after infection, cells were inoculated with an HIV-1 strain, NL43, and culture supernatants were periodically assayed for levels of p24. The datum points are means for triplicate samples with the SD.

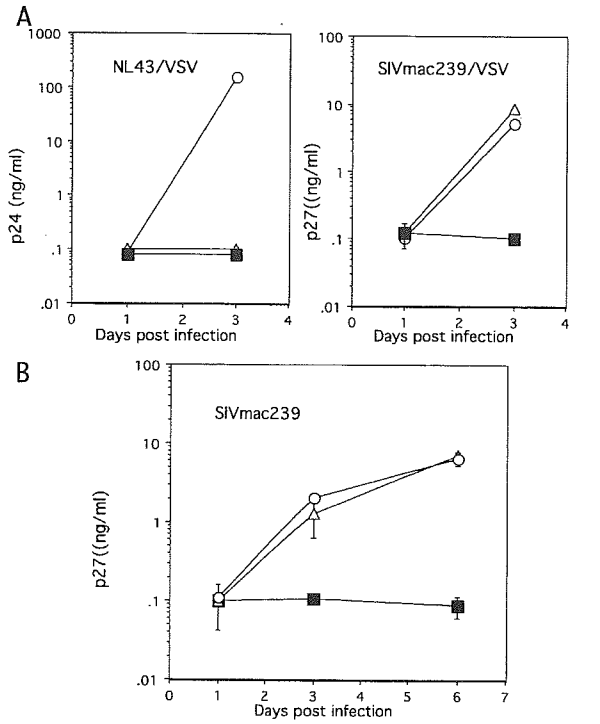

FIG. 3. (A) MT4 (○), HSC-F (△), or CV1 (■) cells were infected with VSV-pseudotyped NL43 or VSV-pseudotyped SIVmac239, and culture supernatants were periodically assayed for levels of p24 or p27. The datum points are means for triplicate samples with the SD. (B) MT4 cells were mock infected (○) or infected with SeV expressing CV1-TRIM5α-tag (■) or HSC-F-TRIM5α-tag (△). At 9 h after infection, cells were inoculated with SIVmac239, and culture supernatants were periodically assayed for levels of p27. The datum points are means for triplicate samples with the SD.

HIV-1 infection, we constructed a recombinant SeV expressing TRIM5α fused with the HA tag in the C-terminal of HSC-F-TRIM5α or CV1-TRIM5α (HSC-F-TRIM5α-tag SeV or CV1-TRIM5α-tag SeV). Human T-cell line MT4 cells were first infected with the SeV expressing TRIM5α-tag (Fig. 2B), incubated at 37°C for 9 h, and then infected with an X4-tropic HIV-1 strain NL43. As can be seen in Fig. 2C, both HSC-F-TRIM5α-tag and CV1-TRIM5α-tag completely inhibited HIV-1 replication, whereas MT4 cells infected with SeV empty vector fully supported HIV-1 replication.

Distinct patterns of restriction for SIVmac among nonhuman primate TRIM5α. In African green monkey CV1 cells, both HIV-1 and SIVmac239 were restricted, whereas only HIV-1 was restricted in cynomolgus monkey HSC-F cells (Fig. 3A). Therefore, we examined whether or not CV1-TRIM5α also could inhibit the replication of SIVmac. In MT4 cells infected with recombinant SeV expressing HSC-F-TRIM5α-tag, the replication of SIVmac239 was not suppressed at all (Fig. 3B), indicating that the HSC-F-TRIM5α showed a similar specificity to rhesus monkey TRIM5α (33). In MT4 cells infected with recombinant SeV expressing CV1-TRIM5α-tag, in contrast, the replication of SIVmac239 was completely suppressed. These results suggested that the distinct sensitivity of African green monkey and cynomolgus monkey cells to HIV-1 and SIVmac infection was, at least partly, determined by TRIM5α.

A small region of 37 amino acid residues in the SPRY domain of CV1-TRIM5α determines SIVmac restriction. A comparison of the human and nonhuman primate TRIM5α sequences showed the presence of a highly variable region in the N-terminal portion of the SPRY domain (Fig. 1A and B). In this region, CV1 and Vero TRIM5α had a 20-amino-acid repetition that was totally absent in HSC-F-TRIM5α. These findings prompted us to test whether this highly variable region of TRIM5α determined the species-specific inhibition of SIVmac infection. We constructed chimeric TRIM5αs from HSC-F-TRIM5α-tag and CV1-TRIM5α-tag by using SphI and BamHI restriction enzyme digestion (Fig. 4A). HSC-F+60tag contained the 242-bp fragment of CV1-TRIM5α in the background of HSC-F-TRIM5α-tag. The reciprocal chimera, CV1-60tag, contained a 182-bp fragment of HSC-F-TRIM5α in the

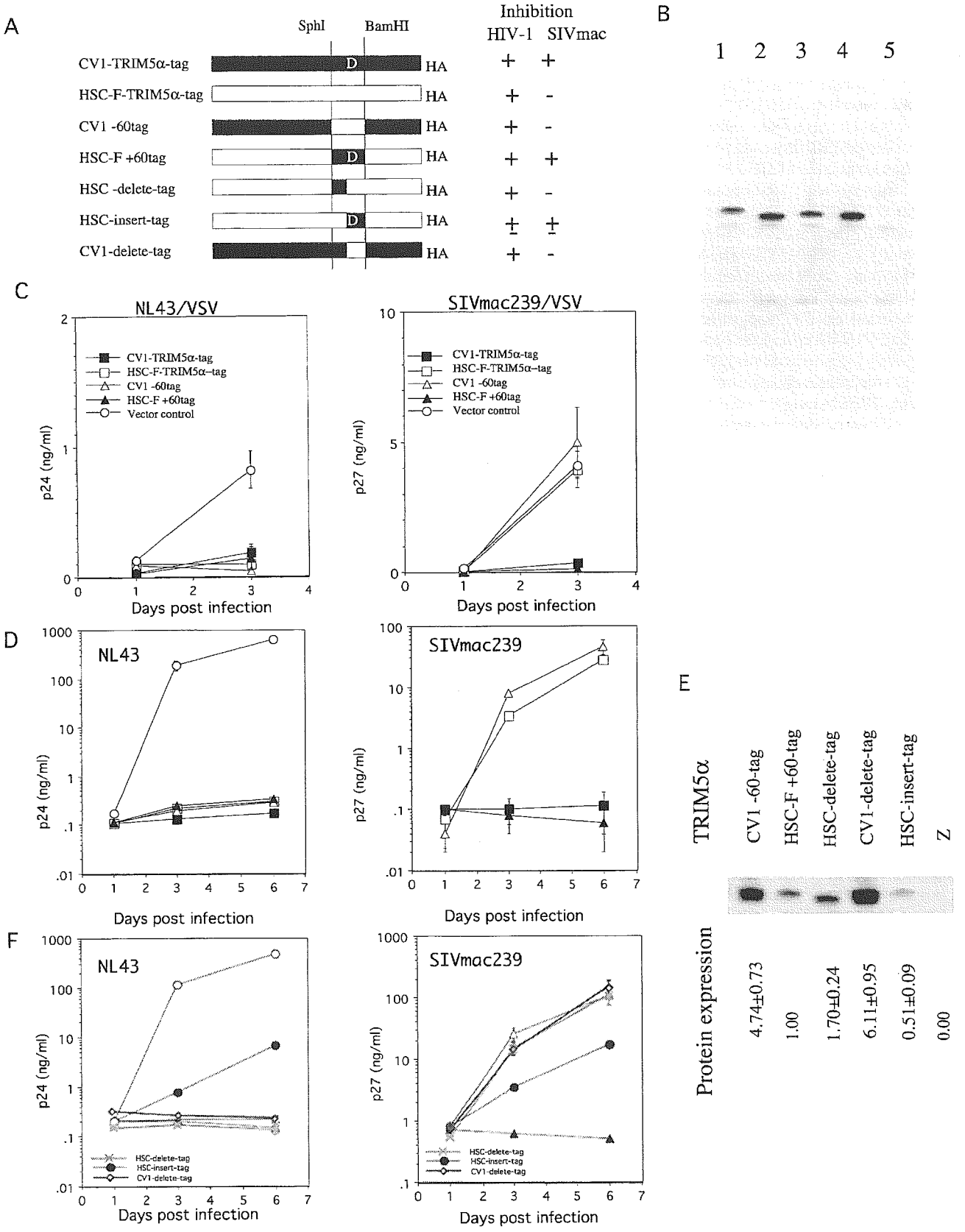

FIG. 4. (A) Schematic representation of chimeric TRIM5α and summary of the results. Filled and open bars denote CV1 and HSC-F sequences, respectively. D, the CV-1-TRIM5α-specific 20-amino-acid duplication. The +, ±, and − symbols denote full, partial, and no

background of the CV1-TRIM5α-tag. In this fragment, the differences between CV1 and HSC-F TRIM5α, including the 20-amino-acid repetition, were located in a small region of 37 amino acid residues (Fig. 1B). Human osteosarcoma C143 cells stably expressing various TRIM5αs (Fig. 4B) were infected with VSV-G-pseudotyped HIV-1 NL43, and levels of P24 in the culture supernatants were assayed periodically. As expected, both chimeric TRIM5αs and parental TRIM5αs clearly inhibited the replication of HIV-1 NL43 (Fig. 4C). When these cells were infected with VSV-G-pseudotyped SIVmac239, the parental CV1-TRIM5α-tag could also inhibit the replication of SIVmac239, whereas CV1-60tag, which contained the 182-bp fragment of HSC-F-TRIM5α in the background of CV1-TRIM5α-tag, could not. Conversely, the parental HSC-F-TRIM5α-tag did not inhibit the replication of SIVmac239 at all, whereas HSC-F+60tag, which contained the 242-bp fragment of CV1-TRIM5α in the background of HSC-F-TRIM5α-tag, clearly inhibited SIVmac239 (Fig. 4C). We obtained the same results as described above when we used SeVs to express parental TRIM5α-tag or chimeras (Fig. 4D). These results indicated that the determinant of the species-specific inhibition of SIVmac239 replication is located in 37 amino acid residues in the SPRY domain of CV1-TRIM5α.

To narrow the SIVmac restriction determinant more precisely, we generated two more chimeric TRIM5αs (Fig. 4A). HSC-delete-tag contained a CV1-derived 17-amino-acid fragment without the 20-amino-acid duplication in the background of the HSC-TRIM5α-tag. HSC-insert-tag contained the HSC-F-derived 17-amino-acid fragment with the CV-1 derived 20-amino-acid duplication in the background of HSC-TRIM5α-tag. Recombinant SeVs expressing these chimeric TRIM5αs were generated and used in the subsequent experiments. Although the expression levels of mRNA of each chimericTRIM5α was virtually identical to each other (data not shown), we observed considerable variations in the levels of TRIM5α protein expression among chimeras (Fig. 4E). The HSC-delete-tag showed slightly higher levels of protein expression than those of HSC-F+60tag. However, HSC-delete-tag failed to inhibit SIVmac replication, whereas it restricted HIV-1 replication as completely as HSC-F+60tag did (Fig. 4F). These results clearly indicated that the CV1-derived 17-amino-acid region alone was not sufficient for SIVmac restriction. On the other hand, HSC-insert-tag partially restricted both HIV-1 and SIVmac, although this chimera showed lower levels of protein expression than other chimeras did (Fig. 4E and F). These results indicated that the CV1-specific 20-amino-acid duplication was important in SIVmac restriction.

To determine whether the CV1-specific 20-amino-acid duplication was indispensable for SIVmac restriction, we generated CV1-delete-tag, which lacked the 20-amino-acid duplication in the CV1-TRIM5α-tag (Fig. 4A). The protein expression level of CV1-delete-tag was comparable to that of CV1-60tag (Fig. 4E), and CV1-delete-tag inhibited HIV-1 replication as completely as CV1-60tag did. However, CV1-delete-tag was shown to lose the ability to inhibit SIVmac infection (Fig. 4F). Taken together, our data clearly indicated that the 20-amino-acid duplication of CV1-TRIM5α was necessary for SIVmac restriction and suggested that the adjacent 17-amino-acid region of CV1-TRIM5α was also necessary to fully restrict SIVmac infection.

HIV-2 GH123 is sensitive to cynomolgus monkey TRIM5α, as well as African green monkey TRIM5α. HIV-2 is closely related to SIVmac (9). We tested whether or not the sensitivity of HIV-2 to various TRIM5αs was similar to that of SIVmac239. C143 cells expressing CV1, HSC-F, and their chimeric TRIM5αs were infected with the VSV-G-pseudotyped HIV-2 strain GH123. Surprisingly, HSC-F-TRIM5α-tag inhibited HIV-2 replication as CV1-TRIM5α-tag had done (Fig. 5). Both chimeric TRIM5αs, CV1-60tag and HSC-F+60tag, also inhibited HIV-2 replication to a similar extent (Fig. 5). These results indicated that HIV-2 strain GH123 was sensitive to cynomolgus monkey TRIM5α despite its high level of sequence homology to SIVmac239.

DISCUSSION

In the present study, we showed that both cynomolgus and African green monkey TRIM5αs could inhibit HIV-1 infection. African green monkey TRIM5α could also inhibit SIVmac infection, whereas cynomolgus monkey TRIM5α could not. Experiments on chimeras of the cynomolgus and African green monkey TRIM5αs unequivocally demonstrated that a small region composed of 37 amino acid residues in the SPRY domain of African green monkey TRIM5α was responsible for restricting the SIVmac infection.

A previous study showed that rhesus monkey TRIM5γ, a splicing variant lacking the SPRY domain, did not suppress HIV-1 infection (33). In the case of TRIM7, the SPRY domain alone was sufficient for binding to its ligand glycogenin (38). Deletion of the entire SPRY domain from TRIM11 also abol-

suppression, respectively. (B) Lysates of C143 cells expressing CV1-TRIM5α-tag (lane 1), HSC-F-TRIM5α-tag (lane 2), HSC-F+60tag (lane 3), CV1-60tag (lane 4), or empty vector (lane 5) were immunoprecipitated by using anti-HA antibody. The resultant immunoprecipitates were visualized by Western blotting with an antibody to HA. A representative result of three independent experiments is shown. (C) C143 cells expressing CV1-TRIM5α-tag (■), HSC-F-TRIM5α-tag (□), CV1-60tag (△), HSC-F+60tag (▲), or empty vector (○) were infected with VSV-pseudotyped NL43 or SIVmac239, and culture supernatants were periodically assayed for levels of p24 or p27. The datum points are means for triplicate samples with the SD. (D) MT4 cells infected with SeV expressing CV1-TRIM5α-tag (■), HSC-F-TRIM5α-tag (□), CV1-60tag (△), HSC-F+60tag (▲), or empty vector (○) were infected with NL43 or SIVmac239, and culture supernatants were assayed for levels of p24 and p27. The datum points are means for triplicate samples with the SD. (E) Lysates of MT4 cells infected with recombinant SeVs expressing CV1-60tag, HSC-F+60tag, HSC-delete-tag, CV1-delete-tag, HSC-insert-tag, or parental Z strain were immunoprecipitated by using anti-HA antibody. Resultant immunoprecipitates were visualized by Western blotting with an antibody to HA. A representative result of three independent experiments is shown. The relative amounts of TRIM5α protein to that of HSC-F+60tag were calculated, and means and SD values of three independent experiments are shown. (F) MT4 cells infected with SeV expressing CV1-60tag (△), HSC-F+60tag (▲), HSC-delete-tag (asterisks with blue lines), HSC-insert-tag (●), CV1-delete-tag (diamonds with red lines), or empty vector (○) were infected with NL43 or SIVmac239, and culture supernatants were assayed for levels of p24 and p27. The datum points are means for triplicate samples with the SD.

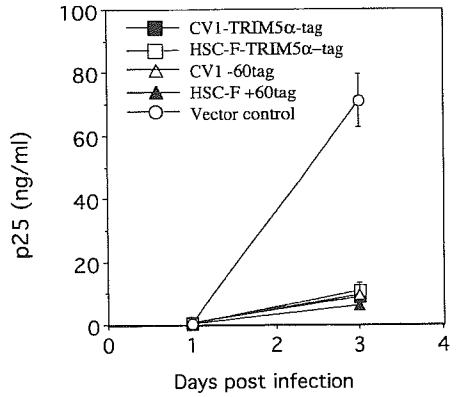

FIG. 5. C143 cells expressing CV1-TRIM5α-tag (■), HSC-F-TRIM5α-tag (□), CV1-60tag (△), HSC-F+60tag (▲), or empty vector (○) were infected with VSV-pseudotyped GH123, and culture supernatants were periodically assayed for levels of p25. The datum points are means for triplicate samples with the SD.

ished its ability to bind Humanin (21). Therefore, it is reasonable to assume that the variable N-terminal region of the SPRY domain of TRIM5α binds to HIV-1 or SIVmac CA protein. This assumption is consistent with the recent findings that in owl monkey cells, HIV-1 infection was restricted by a TRIM5-cyclophilin A fusion protein in which the SPRY domain was replaced with cyclophilin A, since cyclophilin A is a well-known ligand of HIV-1 CA protein (22, 28).

In the attempt to further narrow the SIVmac restriction determinant more precisely, we were able to demonstrate that the African green monkey-specific 20-amino-acid duplication was indispensable for SIVmac restriction and that the adjacent 17-amino-acid region of African green monkey alone was not sufficient. However, HSC-insert-tag carrying the cynomolgus monkey-derived 17-amino acid region with African green monkey-specific 20-amino-acid duplication showed low levels of protein expression and only partial suppression of HIV-1 and SIVmac replication. It is possible that an artificial combination of African green monkey-specific 20-amino-acid duplication with the cynomolgus monkey-derived 17-amino-acid region made TRIM5α molecules unstable. Further studies, including mutational analysis of the African green monkey-specific 17-amino-acid region, are necessary to determine the precise role of this region in SIVmac restriction.

Despite its close similarity to SIVmac, HIV-2 strain GH123 was restricted by cynomolgus monkey TRIM5α, as well as by African green monkey TRIM5α. Although both HIV-2 and SIVmac were considered to come from SIVsm (9), it is possible that HIV-2 has been replicating in the human population in the absence of TRIM5α restriction for a certain period and has lost its ability to escape from cynomolgus monkey TRIM5α. However, it has also been reported that there was a considerable degree of variation in the ability to grow in monkey cells among HIV-2 strains (6, 8, 26). Therefore, it is necessary to examine various HIV-2 strains for their sensitivity to human and monkey TRIM5αs before we can draw a definite conclusion. It would also be interesting to identify specific amino acid changes determining the sensitivity to cynomolgus monkey TRIM5α in viral CA proteins, since nearly 90% of the amino acid residues in SIVmac239 CA protein are conserved in HIV-2GH123.

In CV1 cells, the level of TRIM5 gene expression was ca. 3×10^6 copies/μg of total RNA, a level similar to that observed in other human cell lines examined (data not shown). Although HIV-1 infection was suppressed in hamster TK-ts13 or human C143 cells expressing CV1-TRIM5α, relatively high levels of TRIM5α (nearly 5×10^7 copies/μg of total RNA) appeared to be required for a level of suppression similar to that observed in CV1 cells. One possible explanation for this discrepancy is that certain molecules cooperating with TRIM5α also showed species specificity, and CV1-TRIM5α was not fully supported in hamster and human cells. Because TRIM5 gene products are suspected to be an E3 ubiquitin ligase (35), it is important to identify the E2 ubiquitin-conjugating enzyme interacting with TRIM5α. Alternatively, restriction factors other than TRIM5α may exist in CV1 cells, or certain molecules required for efficient lentivirus infection may be absent in CV1 cells.

After we submitted these findings for publication, small amino acid differences in the SPRY domain between human and rhesus monkey TRIM5αs were reported to determine HIV-1 restriction (27, 34, 37). Our findings are in good agreement with the results of these studies.

ACKNOWLEDGMENTS

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Influence of Glycosylation on the Efficacy of an Env-Based Vaccine against Simian Immunodeficiency Virus SIVmac239 in a Macaque AIDS Model

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The envelope glycoprotein (Env) of human immunodeficiency viruses (HIVs) and simian immunodeficiency viruses (SIVs) is heavily glycosylated, and this feature has been speculated to be a reason for the insufficient immune control of these viruses by their hosts. In a macaque AIDS model, we demonstrated that quintuple deglycosylation in Env altered a pathogenic virus, SIVmac239, into a novel attenuated mutant virus (Δ5G). In Δ5G-infected animals, strong protective immunity against SIVmac239 was elicited. These HIV and SIV studies suggested that an understanding of the role of glycosylation is critical in defining not only the virological properties but also the immunogenicity of Env, suggesting that glycosylation in Env could be modified for the development of effective vaccines. To examine the effect of deglycosylation, we constructed prime-boost vaccines consisting of Env from SIVmac239 and Δ5G and compared their immunogenicities and vaccine efficacies by challenge infection with SIVmac239. Vaccination-induced immune responses differed between the two vaccine groups. Both Env-specific cellular and humoral responses were higher in wild-type (wt)-Env-immunized animals than in Δ5G Env-immunized animals. Following the challenge, viral loads in SIVmac239 Env (wt-Env)-immunized animals were significantly lower than in vector controls, with controlled viral replication in the chronic phase. Unexpectedly, viral loads in Δ5G Env-immunized animals were indistinguishable from those in vector controls. This study demonstrated that the prime-boost Env vaccine was effective against homologous SIVmac239 challenge. Changes in glycosylation affected both cell-mediated and humoral immune responses and vaccine efficacy.

Primate lentiviruses, human immunodeficiency viruses (HIVs), and simian immunodeficiency viruses (SIVs) share common genetic and biological properties. As SIVmac, originally isolated from macaques in primate research centers in the United States, causes AIDS in macaques with remarkable similarities to HIV type 1 (HIV-1) infection in humans, this AIDS monkey model has been utilized to study vaccine development and the pathogenesis of HIV infection (for reviews, see references 10, 14, 17, 43, and 47).

HIV/SIV infection in the host consists of two phases, the primary infection and chronic infection. During the primary infection, extensive viral replication and dissemination of the infection occur. In chronic infection, viral replication continues for a long period, eventually leading to AIDS. Due to the host immune response against the infection, these two phases are separated by a set point at which the viral load reaches its lowest level. The viral loads of the set point and chronic infection are inversely correlated with the control of SIV/HIV infection and predict disease progression (25, 31); however, it remains unclear which host responses determine the viral loads of the set point and chronic infection. Nevertheless, virus-specific immune responses have been implicated in the host's control of the infection. Cellular immunity, such as that shown by cytotoxic T lymphocytes (CTL) and helper T cells, has been reported to correlate with the control of HIV/SIV infection (for reviews, see references 2, 24, 28, and 39). The role of the neutralizing antibody (NAb) in the control of infection and the

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emergence of escape mutants has also been reported previously (7, 16, 51).

Despite these immune responses against HIV/SIV infection, humans and macaques fail to contain the infection due to the virus properties. HIV/SIV infects major target cells, such as $CD4^+$ T cells and macrophages, by binding viral envelope glycoproteins (Env) to cellular surface proteins and CD4 and chemokine receptors (CCR5, CXCR4, or others) on target cells (5, 32). Since viral entry consists of multiple steps (virion binding to these viral receptors, conformational change of Env, and fusion between the virion and the cellular membrane) and the critical parts of Env used in these steps are exposed only during each step, naturally generated antibodies are only partly effective in preventing HIV/SIV infection in their hosts (7, 8). Primary isolates can be neutralized to various degrees by HIV-infected patient serum but not by contemporaneous autologous samples. Consequently, escape mutants against preexisting NAb are selectively replicated (51). Thus, effective NAb is rarely induced in HIV/SIV infection (8, 10). This could partly explain the failure of Env-based vaccine trials against HIV-1 (8, 50).

The heavy glycosylation of Env is a unique feature of HIV/SIV that is distinctive from features of other enveloped viruses and is significantly related to their neutralization-resistant property (8, 29, 44). We therefore assumed that the insufficient immune containment of HIV/SIV might be due to heavy glycosylation in Env and that the removal of some glycans might allow the host to mount a protective immune response against the infection. Thus, we studied the influence of deglycosylation on the replication of SIVmac239 in a T-cell line and created a quintuple deglycosylation mutant of SIVmac239 (Δ5G), which has maximal removal of N-glycans at amino acid residues 79, 146, 171, 460, and 479 in Env and retains a replication capability similar to that of SIVmac239 in phytohemagglutinin-stimulated rhesus peripheral blood mononuclear cells (PBMCs) (36, 40). We then examined the infection of rhesus macaques with Δ5G; although Δ5G was replicated as extensively as SIVmac239 during the primary infection, the subsequent Δ5G infection was restricted to a level less than the detection sensitivity of a plasma viral load assay by 8 weeks postinfection (p.i.), in contrast to high chronic viral replication in SIVmac239 infection. Furthermore, an almost sterilizing immunity against SIVmac239 was induced in Δ5G-infected animals (36). Interestingly, another quintuple-deglycosylation-mutation strain with mutations at amino acid residues 146, 156, 184, 244, and 247 in Env was created (44) and was demonstrated to share common features with Δ5G in viral replication in animals and in functions as an attenuated vaccine (20). Since these two viruses share only one deglycosylation mutation and other mutations distributed differently in surface envelope protein gp120 (SU), these two studies suggest that heavily glycosylated Env determines the pathogenicity of HIV/SIV.

To dissect the mechanism for notable containment of Δ5G infection after primary infection, we hypothesized that the Env of Δ5G, a viral protein that differs from that in SIVmac239, might elicit protective immunity against SIVmac239, because deglycosylation in Env might alter antigenic properties such as B-cell and T-cell epitopes and enhance the protective immunity against SIVmac239. For this purpose, we immunized animals with Env of Δ5G (Δ5G Env) or Env of SIVmac239 (the wild type; wt Env), and examined the effect of these vaccinations against SIVmac239 infection.

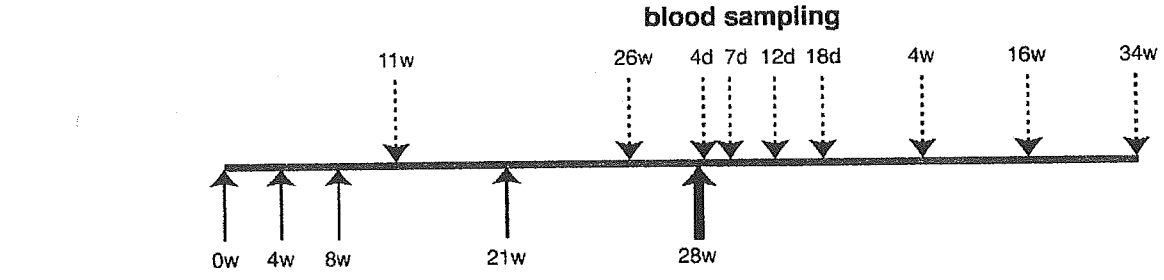
MATERIALS AND METHODS

Generation of SU DNA vaccines. DNA vaccine plasmids expressing SIV mac239 SU or Δ5G SU, pJWSUmac239 and pJWSUmacΔ5G, were constructed using the expression vector pJW4303 (45). To produce secreted SU efficiently, the native signal sequence in the SIVmac239 SU gene was replaced with the human tissue plasminogen activator signal in plasmid pJW4303, and a termination codon was created at the cleavage site for SU transmembrane (TM) protein (9). An SIVmac239 SU or Δ5G SU DNA sequence was amplified with a pair of primers, SUmacA (5′-TGT**GCTAGC**TATGTCACAGTCTTTTATGGTGTAC-3′) and SUmacB (5′-CCA**GGATCC**TATTACCTCTTCACATCTGTGGGGG C-3′). The SUmacA primer consisted of nucleotides (nt) 6923 to 6955 of the SIVmac239 sequence (GenBank accession number M33262) and the boldface nucleotides, which were changed to create a NheI site; primer SUmacB consisted of nt 8412 to 8381 and the boldface nucleotides, which were changed to create a BamHI site, and the underlined nucleotides, which generated tandem termination codons. The PCR-amplified fragments were digested with NheI and BamHI and cloned into the NheI- and BamHI-digested eukaryotic expression vector pJW4303 to yield pJWSUmac239 and pJWSUmacΔ5G. These plasmids were prepared using a Plasmid Mega kit (QIAGEN, Tokyo, Japan).

Generation of Env vaccinia vaccines. Recombinant vaccinia viruses expressing Env of SIVmac239 or Δ5G, WRvvmac239 or WRvvΔ5G, respectively, were constructed using a vaccinia virus WR strain (WRvv) as described previously (15). To excise the entire coding region of the *env* gene from the cloned SIV plasmid, BamHI and SmaI sites were introduced by in vitro mutagenesis at 5′- and 3′-end-flanking sites of the *env* gene, respectively. Primer B-6808 (5′-GAA AGAGAAGAAGGATCCCGAAAAAGG-3′) consisted of nt 6796 to 9822 and the underlined mutations of the BamHI site; S-9537 (5′-TATGAATACTCCC GGGAGAAACCC-3′) consisted of nt 9527 to 9550 and the underlined mutations of the SmaI site. DNA fragments containing the *env* gene of SIVmac239 or Δ5G were isolated by digesting the mutated plasmids with BamHI and SmaI and were cloned into the SmaI- and BamHI-digested vaccinia virus vector plasmid pNZ68K2. To transfer the *env* gene from a recombinant plasmid to WRvv, the standard homologous recombination method using CV-1 cells was performed. Env expression in the recombinant vaccinia virus was confirmed by immunoprecipitation. The function of Env was confirmed by CD4- and CCR5-dependent fusion activity. The recombinant Env-expressing vaccinia viruses obtained were propagated and titrated in CV-1cells. The two recombinant viruses were propagated with similar kinetics in CV-1 cells.

Expression of SU-expressing plasmids and Env-expressing vaccinia virus in vitro. CV-1 cells were transfected with equal amounts of the following SU-expressing plasmids: pJWSUmac239, pJWSUmacΔ5G, or the vector pJW4303. Secreted SU metabolically labeled with ^{35}S protein labeling mix (PerkinElmer, Boston, MA) in culture supernatant was concentrated, immunoprecipitated with plasma from SIVmac239-infected monkeys, and then analyzed by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) as described previously (40). To examine Env-expressing vaccinia viruses, CV-1 cells were infected with WRvvmac239, WRvvΔ5G, or WRvv at a multiplicity of infection of 10, metabolically labeled with ^{35}S protein labeling mix overnight, lysed, immunoprecipitated with plasma from SIVmac239-infected monkeys, and then analyzed by SDS-PAGE as described for the expression of SU-expressing plasmids.

Animals, immunization, and challenge. Twelve juvenile rhesus macaques from Myanmar or Laos that were seronegative for SIV, simian T-cell lymphotropic virus, B virus, and type D retroviruses were used. As the polymorphism of major histocompatibility complex (MHC) genes influenced cellular immune responses against SIV/HIV infection, MHC II haplotypes and alleles of the macaques were determined (data not shown). All animals were housed in individual cages and maintained according to the rules and guidelines for experimental animal welfare stated by the National Institute of Infectious Diseases. As shown in Fig. 1, the 12 animals were divided into three immunization groups of four animals each: the SIVmac239 (wt)-Env immunization group (Mm0005, Mm0007, Mm0010, Mm0012), the Δ5G Env immunization group (Mm0001, Mm0002, Mm0003, Mm0009), and the vector control immunization group (Mm0004, Mm0006, Mm0008, Mm0011). All animals were inoculated with 1 mg of plasmid DNA in 1 ml of saline, one into each quadriceps femoris at 0, 4, and 8 weeks after the initial prime immunization (weeks p.p.). The boost consisted of 5×10^7 PFU of vaccinia virus in 1 ml of phosphate-buffered saline (PBS), administered in two 0.1-ml intradermal inoculations, one into the skin of each femur, and two 0.4-ml inoculations, one into each quadriceps femoris at 21 weeks p.p. All animals were



Vaccine Groups:	DNA	Vaccinia boost	SIV challenge
wt-Env (n=4)	pJWSUmac239 (SU: gp120)	WRvvENVmac239 (Env: gp160)	SIVmac239
Δ5G-Env (n=4)	pJWSUΔ5G (SU: gp120)	WRvvENVΔ5G (Env: gp160)	SIVmac239
vector control (n=4)	pJW4303	WRvv	SIVmac239

FIG. 1. Outline of immunization, challenge infection, and blood sampling. Twelve juvenile rhesus macaques were divided into three immunization groups of four animals each: the wt-Env immunization group (Mm0005, Mm0007, Mm0010, and Mm0012), the Δ5G Env immunization group (Mm0001, Mm0002, Mm0003, and Mm0009), and the vector control immunization group (Mm0004, Mm0006, Mm0008, and Mm0011). Animals were inoculated with a DNA vaccine (pJWSUmac239 for the wt-Env vaccine group, pJWSUΔ5G for the Δ5G Env vaccine group, and pJW4303 for the vector control group) at 0, 4, and 8 weeks p.p. The boost vaccine consisted of vaccinia virus (WRvvENVmac239 for the wt-Env vaccine group, WRvvENVΔ5G for the Δ5G Env vaccine group, and the WR strain for the vector control group) administered at 21 weeks p.p. All animals were challenged with 10 $TCID_{50}$ of SIVmac239 intravenously at 28 weeks p.p. w, weeks; d, day.

challenged with 10 50% tissue culture infective doses ($TCID_{50}$) of SIVmac239 intravenously at 28 weeks p.p.

Viral load measurement. To monitor SIV infection, the plasma viral load was measured by the real-time-PCR method described previously (36). Viral RNA was isolated from plasma from the infected animals using a commercial viral-RNA isolation kit (PE Applied Biosystems, Urayasu, Japan). SIV *gag* RNA was amplified and quantified using a commercial RNA reverse transcription (RT)-PCR kit (TaqMan EZ RT-PCR; PE Applied Biosystems) with the two *gag* primers, namely, the forward primer 1224F (5′-AATGCAGAGCCCCAAGAAGAC-3′), the reverse primer 1326R (5′-GGACCAAGGCCTAAAAAACCC-3′), and TaqMan probe 1272T (6-carboxyfluorescein-5′-ACCATGTTATGGCCAAATGCCCAGAC-3′-6-carboxymethylrhodamine). Purified viral RNA (10 μl) was reverse transcribed and amplified in a MicroAmp optical 96-well reaction plate (PE Applied Biosystems) according to the manufacturer's instructions and with the following thermal cycle conditions: 1 cycle of three sequential incubations (50°C for 2 min, 60°C for 30 min, and 95°C for 5 min) and then 50 cycles of amplification (95°C for 5 s, 62°C for 30 s) in a 7000 Prism sequence detection system (PE Applied Biosystems). In vitro RNA transcripts were quantified by optical density at 260 nm (OD_{260}) measurement and branched DNA assay for SIV viral RNA (Bayer Diagnostics, Tarrytown, N.Y.). RNA equivalent to 10 to 10^7 copies per reaction was used as the standard for each assay. The detection sensitivity of plasma viral RNA using this method was 1,000 copies/ml.

Flow cytometry. CD4 depletion was monitored by measuring the percentage of $CD4^+$ T cells, memory cells (CD29 high $CD4^+$) T cells (48) in PBMCs. PBMC samples were purified from a citrate anticoagulant containing blood using standard Ficoll-Hypaque gradient centrifugation. For flow cytometry, 2×10^5 PBMCs were reacted with fluorescein isothiocyanate or phycoerythrin-labeled antibodies (anti-human CD4, Nu-Th/I [Nichirei, Tokyo, Japan]; anti-human CD8, Leu2a [Becton Dickinson, San Jose, CA]; anti-human CD29, 4B4 [Coulter, Miami, FL]; anti-monkey CD3, FN-18 [Biosource, Camarillo, CA]; and anti-human CD20, Leu16 [Becton Dickinson, San Jose, CA]) as previously described (36, 37, 48).

Peptides. Overlapping peptides were synthesized by Emory University, Microchemical Facility, Winship Cancer Center (Atlanta, GA.). All SIVmac239 viral proteins except Env, Gag, Pol, Vif, Vpr, Vpx, Tat, Rev, and Nef were covered by consecutive 20-mer peptides overlapped by 12 amino acids. Env of SIVmac239 was covered by 72 consecutive 25-mer peptides overlapped by 13 amino acids. Peptides were dissolved in PBS with 10% dimethyl sulfoxide (Sigma Chemical, St. Louis, Mo.).

rSeV. Recombinant Sendai viruses (rSeV) expressing SIVmac239 Gag, SU, or Δ5G SU were used to infect herpesvirus papio-transformed B-lymphoblastoid cell lines (B-LCLs) to prepare autologous B-LCLs presenting these viral antigens. rSeV Gag expressing unprocessed SIVmac239 Gag and p55 (22, 23) and rSeV SU and rSeV/Δ5G SU expressing wt SU and Δ5G SU were constructed as described previously (52) and were also used to infect autologous B-LCLs.

Anti-SIV ELISA. A 1:100 dilution of each plasma sample in PBS (pH 7.4) containing a blocking reagent (Dainippon Seiyaku, Osaka, Japan) was assayed for SIV-specific antibody by using a standard enzyme-linked immunosorbent assay (ELISA) technique with 96-well plates precoated with SIVmac239 virion lysate. The OD_{492} was measured using a microplate reader (range of absorbance with linearity, 0 to 3.0; Tecan Japan, Tokyo, Japan) and utilized as a relative measurement of the antibody titer.

ELISPOT assay. Virus-specific $CD4^+$ T cells and $CD8^+$ T cells in PBMCs were measured using a monkey γ-IFN ELISPOT assay kit (U-CyTech, Utrecht, The Netherlands).

Cryopreserved PBMCs were thawed and cultured overnight in R-10 medium (RPMI 1640 [Sigma] supplemented with 10% heat-inactivated, defined fetal bovine serum [HyClone, Logan, Utah], 55 μM 2-mercaptoethanol, 50 U/ml penicillin, and 50 μg/ml streptomycin). PBMCs were subjected to the depletion of $CD4^+$ cells with magnet beads coated with anti-human CD4 Ab (Dynal ASA, Oslo, Norway) or subjected to the depletion of $CD8^+$ cells with magnet beads coated with anti-human CD8 Ab (Miltenyi Biotec, Bergisch Gladbach, Germany). Depletion of $CD4^+$ or $CD8^+$ cells from PBMCs was confirmed by flow cytometry. Using this depletion method, more than 95% of $CD4^+$ or CD8 cells were removed from PBMCs. These PBMCs were used for ELISPOT assay for virus-specific $CD8^+$ T cells and virus-specific $CD4^+$ T cells. Virus-specific stimulation of T cells was performed with autologous B-LCLs pulsed with pooled peptides for Pol, Vif, Vpx, Vpr, Tat, Rev, and Nef or B-LCLs infected with an rSeV for Gag, wt Env, and Δ5G Env. B-LCLs were incubated with pooled peptides corresponding to each viral protein at a final concentration of 2 μg/ml or infected with rSeV at a multiplicity of infection of 10 at 37°C overnight. Peptide-pulsed or infected B-LCLs were inactivated with long-wave UV irradiation (19) in the presence of 10 μg/ml psoralen (Sigma) for 10 min at a distance of 3.5 cm from a UV light, washed three times with R-10, and then used as stimulators in an ELISPOT assay. $CD4^+$ or $CD8^+$ cell-depleted PBMCs were cultured with these stimulators in an anti-γ-IFN Ab-coated ELISPOT plate (U-CyTech) overnight according to the protocol for the kit. Spots on the ELISPOT plate were imaged using an Olympus model SZX12 microscope

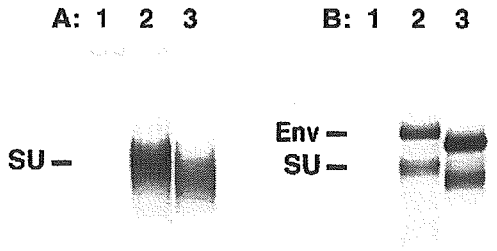


FIG. 2. Expression of SU and Env by SU-expressing DNA vaccines and Env-expressing vaccinia viruses. A: SU secreted in supernatant from CV-1 cells transfected with SU-expressing plasmids. Lane 1, pJW4303 vector; lane 2, pJWSUmac239; lane 3, pJWSUmacΔ5G. B: Env in cell lysates of CV-1 cells infected with recombinant vaccinia viruses. Lane 1, WRvv; lane 2, WRvvmac239; lane 3, WRvvΔ5G.

(Olympus, Tokyo, Japan) equipped with a digital camera, PDMCIe/OL (Polaroid, Cambridge, MA), and analyzed using a personal computer with MAC SCOPE version 2.61 (Mitani Corporation, Toyama, Japan). The results were calculated as numbers of spot-forming cells (SFC) per million PBMCs after subtraction of the background.

Neutralization assay. The original protocol of this neutralization assay was reported by Means et al. (29). Plasma that was heat inactivated at 56°C for 30 min was serially diluted and incubated with a fixed concentration of SIVmac239, Δ5G, or a macrophage-tropic SIV, 239/envMERT, at room temperature for 1 h. CEMx174/SIVLTR-SEAP cells were added to the mixture and then incubated at 37°C for 3 days. Secreted alkaline phosphatase activity in the culture supernatant was measured using a Phospha-Light System (Applied Biosystems). Chemiluminescence was detected with a Wallac Microbeta plate reader.

Statistical analysis. Statistical analysis was based on the Mann-Whitney test and performed using GraphPad Prism 4.0 software.

RESULTS

Experimental design. We adopted a DNA prime-vaccinia virus boost regimen to immunize rhesus macaques with wt Env or Δ5G Env as shown in Fig. 1. Twelve macaques were immunized at 0, 4, and 8 weeks after the initial prime immunization (weeks p.p.) with one of three different DNA expression plasmids ($n = 4$): pJWSUmac239 expressing SU of SIVmac239, pJWSUΔ5G expressing SU of Δ5G, or the vector pJW4303. At 21 weeks p.p., all animals were boosted with recombinant WR vaccinia viruses expressing the respective Env proteins: vaccinia virus expressing Env of SIVmac239, vaccinia virus expressing Env of Δ5G, or vaccinia virus (Fig. 1).

Expression of SU DNA plasmids and Env vaccinia viruses in vitro and in animals. Although Δ5G replicated similarly to wild-type SIVmac239 in animals (36), quintuple deglycosylation might affect the expression of SU in a plasmid vector and the expression of Env in the vaccinia virus vector. Thus, we examined the expression of these vaccines in CV-1 cells. SU expressions in the wild-type plasmid (pJWSUmac239) and in the deglycosylated SU plasmid (pJWSUmacΔ5G) were at similar levels (Fig. 2A). The expression and processing of Env in the wild type (WRvvENVmac239) and in the deglycosylated Env mutant vaccinia virus (WRvvENVΔ5G) were also at similar levels (Fig. 2B). The reduced molecular size of the proteins due to deglycosylation was confirmed by PAGE (Fig. 2). As the amount of secreted SU in the supernatant by DNA transfection was comparable to that of Env in the cell lysate from CV-1 cells infected with WRvvEnv, a high expression of SU was achieved in a *rev*-independent manner by the pJW403 expression plasmid as described previously (9).

The expression of Env vaccines in the immunized animals was indirectly estimated by Env-specific antibody responses measured by a peptide ELISA using overlapping Env peptides. Env peptide-specific Ab was detected from 11 weeks p.p. after immunization with DNA vaccines, whereas there was no significant difference in the titers and the specificity of the responses between the two vaccine groups (data not shown), suggesting similar amounts of Env expressed in animals immunized with either Env vaccine. To examine the protective effect of the Env vaccines, all animals were challenged with 10 $TCID_{50}$ of SIVmac239 intravenously at 28 weeks p.p.

Cellular immune responses elicited by Env vaccines. The DNA prime-vaccinia virus boost regimen has been used in many studies, has successfully induced a high frequency of virus-specific $CD8^+$ T cells in macaques, and has conferred protective immunity against chimeric simian/human immunodeficiency virus (SHIV) (3, 27, 45). We therefore examined the vaccine-induced Env-specific T-cell responses by IFN-γ ELISPOT assay. Since deglycosylation in Env might change T-cell epitopes in SIVmac239, we measured the wt-SU and Δ5G SU-specific T-cell response by using autologous B-LCLs infected with recombinant Sendai viruses expressing either wt SU and/or Δ5G SU, respectively.

Although there was a tendency for more ELISPOT-positive cells to be observed by homologous SU than heterologous SU, comparable results were obtained by both assays (Fig. 3A and B). As vaccinated animals were challenged with SIVmac239, the results from the wt-SU assay were subsequently used to assess the SU-specific immune response. Immunization with the DNA vaccine induced only marginal SU-specific $CD8^+$ T cells or $CD4^+$ T cells at 11 weeks p.p.; however, boost immunization with recombinant WR vaccinia virus significantly increased SU-specific $CD8^+$ T cells and $CD4^+$ T cells in PBMCs at 26 weeks p.p. (Fig. 3A, B, and C). Notably, SIVmac239 Env (wt Env) induced twofold more SU-specific CD8 T cells (mean, 770 SFC per million PBMCs; range, 540 to 880) responding to wt SU than Δ5G Env (mean, 320; range, 110 to 400) ($P = 0.029$) (Fig. 3A and C). Similarly, twofold more SU-specific $CD4^+$ T cells were observed in wt-Env vaccinees (mean, 1,260; range, 840 to 1,710) than in Δ5G Env vaccinees (mean, 680; range, 150 to 1,260) at 26 weeks p.p. ($P = 0.11$) (Fig. 3B and C). Thus, a twofold-greater number of both SU-specific $CD4^+$ T cells and $CD8^+$ T cells were induced in SIVmac239 Env vaccinees than in Δ5G Env vaccinees at 26 weeks p.p. In vector controls, only negligible SU-specific $CD4^+$ T cells and $CD8^+$ T cells were detected in PBMCs at 26 weeks p.p. (Fig. 3A and B).

Humoral immune response elicited with Env vaccines. The anti-Env Ab titer was examined by SIVmac239 virion lysate ELISA. Anti-SIV Ab was detected in both wt-Env vaccinees and Δ5G Env vaccinees after an rVV boost (Fig. 4) (26 weeks p.p.). Anti-SIV Ab titers were comparable between the two vaccine groups.

Next, we examined the NAb against either SIVmac239, Δ5G, or a macrophage-tropic mutant, 239env/MERT (33, 35), in the two vaccine groups. Macrophage-tropic SIVs were highly susceptible to neutralization by plasma from most SIV-infected macaques (29), whereas SIVmac239 was highly resistant to neutralization as were most clinical isolates of HIV-1

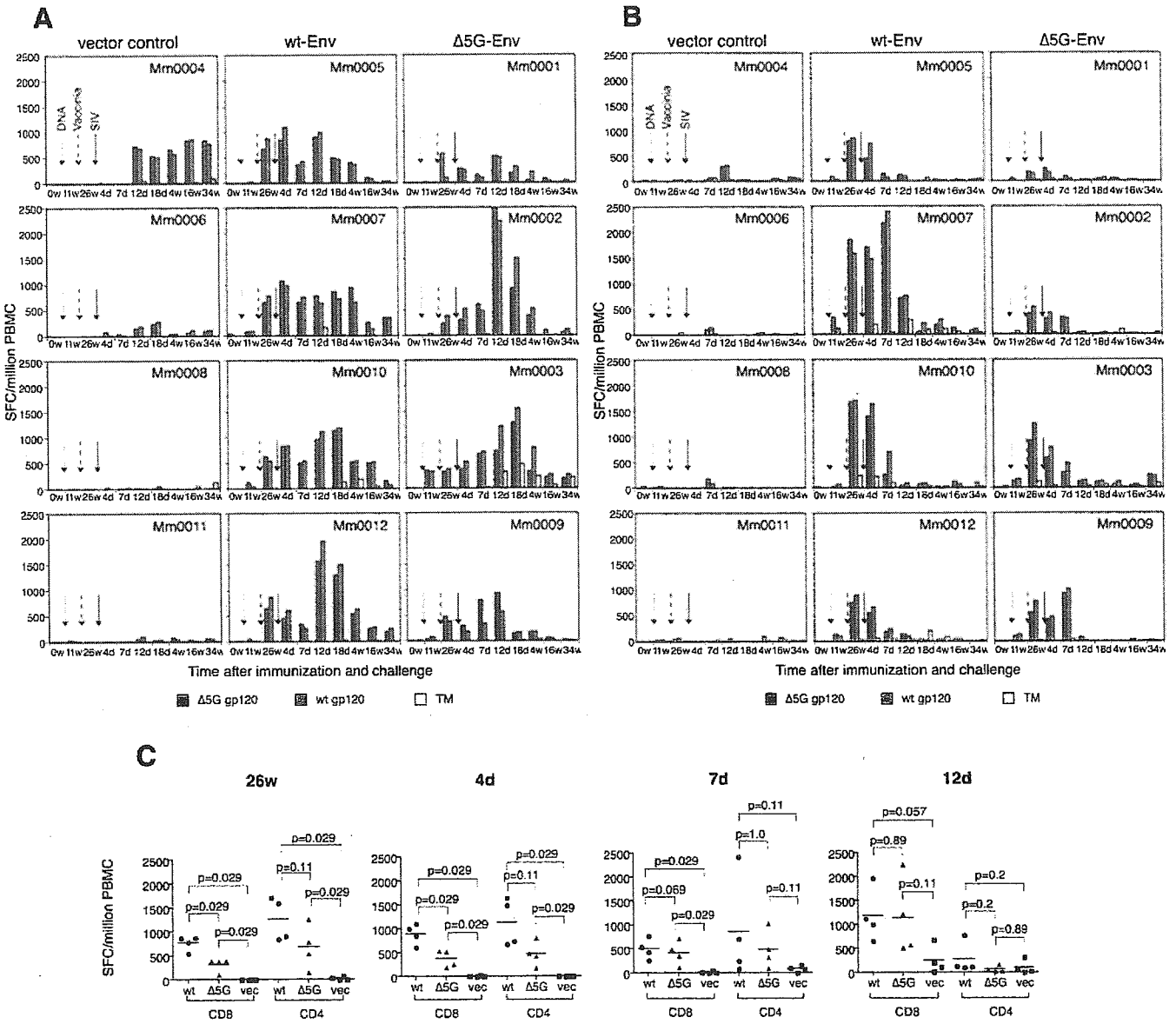


FIG. 3. Env-specific $CD4^+$ T-cell and $CD8^+$ T-cell responses in 12 macaques. A: Env-specific $CD8^+$ T cells in PBMCs were measured by ELISPOT assay for IFN-γ in three groups. B: Env-specific $CD4^+$ T cells in PBMCs were measured by ELISPOT assay for IFN-γ in three groups. ELISPOT results are colored as follows: Δ5G SU-specific T cells (red), wt-SU-specific T cells (green), and TM-specific T cells (yellow). Arrows with a dotted line, arrows with broken line, and arrows with a solid line indicate the time of the third DNA vaccination at 8 weeks p.p., the time of the vaccine boost at 21 weeks p.p., and the time of SIVmac239 challenge at 28 weeks p.p., respectively. C: Comparison of SU-specific $CD8^+$ T cells and $CD4^+$ T cells in PBMCs among the wt-Env vaccine group, the Δ5G Env vaccine group, and the vector control group at 26 weeks p.p. and 4, 7, and 12 days p.i. The numbers of SFC responding to SIVmac239 SU were used to compare the effects of the two vaccines. w, weeks; d, days.

(21, 29, 30). Plasma at 26 weeks p.p. from all immunized animals failed to neutralize not only SIVmac239 but also a multiple-deglycosylation-mutation strain, Δ5G (Table 1); in contrast, these plasma specimens did neutralize 239env/MERT. Furthermore, a marked difference was observed between the two vaccine groups. The NAb titer in the wt-Env vaccine group was eightfold higher than in the Δ5G Env vaccine group (Table 1). The difference of this immune response between the two vaccine groups was significant ($P = 0.029$).

SIV replication in Env-immunized animals. As described above, wt-Env vaccine and Δ5G Env vaccine induced different magnitudes of virus-specific cellular and humoral immunity in macaques. To examine the effect of the two vaccines, we challenged the vaccinated animals with SIVmac239. Viral loads in vector controls were mostly consistent with our previous results with SIVmac239-infected rhesus macaques (36, 48). The mean peak viral load at 2 weeks p.i. was 1.4×10^7 copies/ml, with a range of 0.5×10^7 to 2.2×10^7 copies/ml. Viral loads in chronic infection diverged into two patterns (Fig. 5A). Subsequent to the set point at 20 weeks p.i., the viral loads in three animals increased more than 10^4 copies/ml. In contrast, viral loads in one animal (Mm0011) remained as low as 1,000 copies/ml up to 45 weeks p.i.

Compared with the vector controls, viral loads in wt-Env

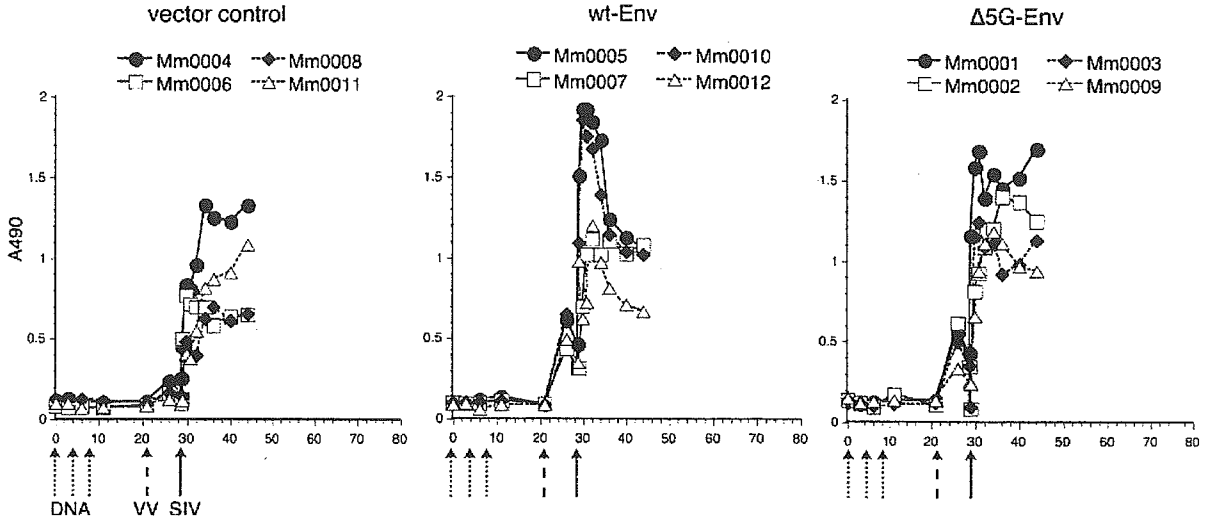


FIG. 4. Humoral immune response during immunization and after challenge infection. The OD_{492} was used as a relative measurement of anti-SIV ELISA antibody titer.

vaccinees were markedly reduced (Fig. 5B). Peak viral loads at 2 weeks p.i. (mean, 1×10^6 copies/ml; range, 0.8×10^6 to 1.2×10^6 copies/ml) were 1-log lower than those in the vector controls. Furthermore, viral loads decreased to as low as 1,000 copies/ml by 8 to 20 weeks p.i., remaining low until autopsy at 45 weeks p.i.

Unexpectedly, viral loads in the Δ5G Env vaccine group resembled those in vector controls (Fig. 5C). Peak viral loads (mean, 2.4×10^6 copies/ml; range, 0.9×10^6 to 4.2×10^6 copies/ml) were slightly lower than those in vector controls. Set points and viral loads in the chronic phase were similar to those of vector controls.

In summary, as shown by the mean viral loads in primary and chronic infection (Fig. 5D) and statistical analysis (Fig. 5E), the effects of vaccination differed between the wt-Env vaccine and Δ5G Env vaccine. In the effect on primary infection (up to 6 weeks p.i.), wt-Env vaccination decreased viral loads more extensively and significantly than Δ5G Env vaccination (P = 0.029 versus P = 0.057); however, in chronic infection (viral loads after 8 weeks p.i.), significant reductions in viral loads compared with those in vector controls were seen only in the wt-Env vaccine group and not the Δ5G Env vaccine group (Fig. 5E). Collectively, wt-Env vaccination induced significantly effective immunity to control SIVmac239 infection, whereas Δ5G Env vaccination induced a marginal effect seen only in primary and not in chronic infection.

TABLE 1. Neutralizing-antibody titers in the vaccinated macaques at 26 weeks p.p.

Vaccine	Animal	Neutralizing-antibody titer[a]			Mean[b]
		SIVmac239	Δ5G	239/envMERT	
wt-Env	Mm0005	<20	<20	800	
	Mm0007	<20	<20	400	
					400
	Mm0010	<20	<20	400	
	Mm0012	<20	<20	200	
Δ5G-Env	Mm0001	<20	<20	100	
	Mm0002	<20	<20	20	
					50
	Mm0003	<20	<20	100	
	Mm0009	<20	<20	50	

[a] Reciprocal of the dilution of plasma giving 50% inhibition of SIV replication.
[b] The difference in NAb levels between the two vaccine groups was significant (P = 0.0029).

$CD4^+$ T-cell subsets in PBMCs. CD4 cell depletion is a primary manifestation indicating immune disorder in HIV/SIV infection. As CD4 depletion results from HIV/SIV infection in lymphatic tissue, it correlates with the extent of viral replication. Accordingly, viral loads were correlated mostly with CD4 depletion (Fig. 5 and 6A). Despite fluctuations due to immunizations and the challenge infection, the percentage of $CD4^+$ T cells in wt-Env-immunized animals in the chronic phase recovered to the levels at the initiation of the experiment. By contrast, in vector controls and Δ5G Env vaccinees, the percentage of $CD4^+$ T cells decreased in the chronic phase. Among them, an extensive decrease in $CD4^+$ T cells occurred in animals with high viral loads in the chronic phase (Mm0001, Mm0008, and Mm0009) (Fig. 5 and 6A). However, in the other animals, the levels of $CD4^+$ T cells remained as before the challenge (Mm0003, Mm0011).

A subset of $CD4^+$ CD29 high cells, approximately corresponding to memory $CD4^+$ T cells, is useful for diagnosing a deterioration in the immune function in animals with AIDS (26, 38, 48). Although this parameter usually correlates with the percentage of $CD4^+$ T cells, remarkable differences were noted between two Env vaccine groups after the challenge infection. First, all animals in the wt-Env vaccine group showed an increased percentage of this subset in the chronic phase (Fig. 6B). Second, three of the Δ5G Env vaccinees had a marked decrease after the challenge infection (Mm0001, Mm0002 and Mm0009), whereas the remaining animal (Mm0003) showed an increased percentage of this subset. In

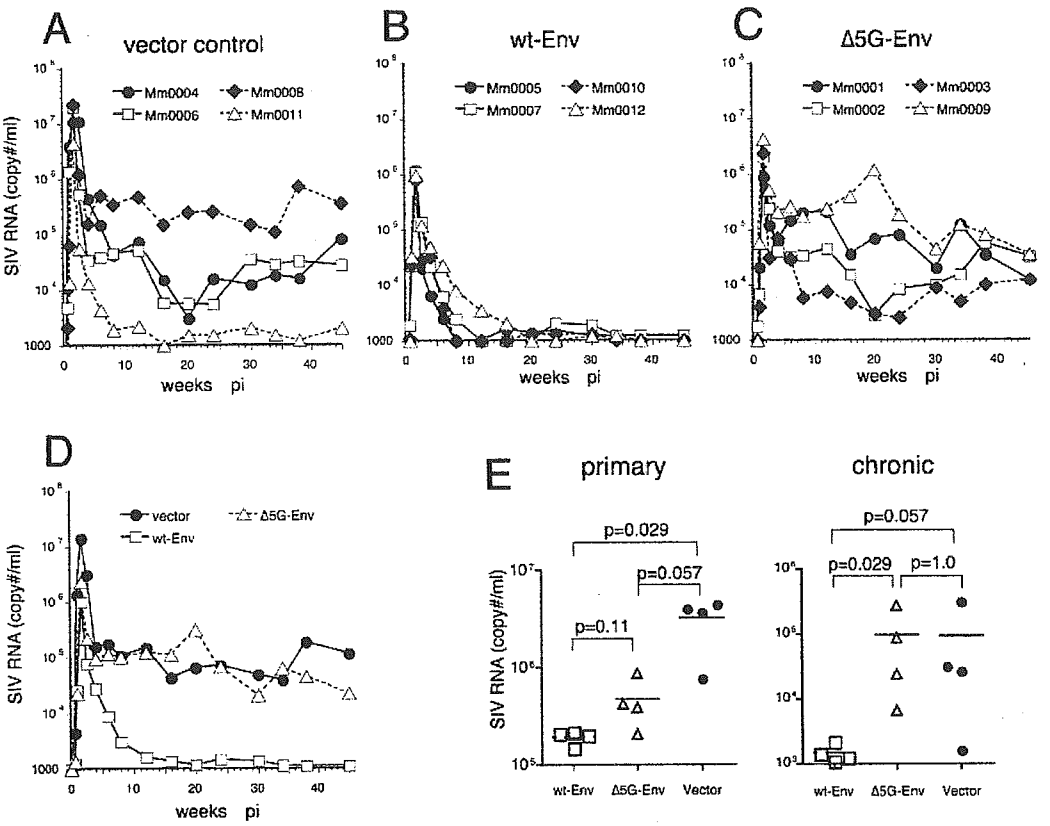


FIG. 5. Plasma viral loads after SIVmac239 challenge infection. Plasma viral load was measured by real-time PCR with a detection limit of 1,000 copies/ml. A: wt-Env vaccine group; B: Δ5G Env vaccine group; C: vector controls; D: comparison of viral loads among three groups; E: comparison of viral loads during the primary infection (5 days to 6 weeks p.i.) and chronic infection (8 weeks to 45 weeks p.i.) among three groups. Viral load was determined by averaging over a period of time.

vector controls, this subset remained in the range before the challenge infection in all animals but one (Fig. 6B).

Env-specific-T-cell immunity after the challenge infection. The magnitude of Env-specific T cells after the challenge infection is assumed to be influenced not only by vaccination but also by viral replication. Namely, SU-specific T cells at 4 days p.i. and those at 12 days p.i. were likely influenced by the former and the latter respectively. The magnitudes of SU-specific $CD4^+$ T cells and $CD8^+$ T cells at 4 days p.i. were comparable to those before challenge at 26 weeks p.p. (Fig. 3A and B); therefore, twofold-more SU-specific $CD8^+$ T cells and $CD4^+$ T cells were present in wt-Env vaccinees than in Δ5G Env vaccinees up to 4 days p.i. (Fig. 3C). However, this difference in the magnitudes of SU-specific $CD8^+$ T and $CD4^+$ T cells was not sustained at 7 and 12 days p.i. (Fig. 3C). Present with robust viral replication in primary infection, SU-specific $CD4^+$ T cells immediately decreased to an undetectable level at 12 days p.i. In contrast, SU-specific $CD8^+$ T cells increased (Fig. 3A and B). Subsequently, SU-specific $CD8^+$ T cells gradually decreased to very low or undetectable levels by 34 weeks p.i. (Fig. 3A). Thus, vaccine-induced SU-specific $CD8^+$ T and $CD4^+$ T cells were sustained only for a short period of time after challenge infection in both Env vaccine groups.

SIV-specific T-cell immunity after challenge infection. Despite an Env vaccination, robust SIV infection occurred shortly after the challenge infection (Fig. 5B and C). Consequently, SIV-specific $CD8^+$ T cells and $CD4^+$ T cells were elicited not only in vector controls but also in Env vaccine groups (Fig. 7A and B). To examine the effect of these SIV-specific T cells on the control of SIV infection, all animals were divided into SIV infection-controlled (controlled) and SIV infection-uncontrolled (uncontrolled) animals. Viral loads in chronic infection and the percentage of $CD4^+$ cells in PBMCs were used to classify the animals as controlled or uncontrolled (Fig. 6A). All animals in the wt-Env vaccine group, Mm00011 in vector controls, and Mm0003 in the Δ5G Env vaccine group were grouped as control animals. The remaining animals, Mm0004, Mm0006, and Mm0008 in vector controls and Mm0001, Mm0002, and Mm0009 in the Δ5G Env vaccine group were grouped as uncontrolled animals. Notably, SIV-specific $CD4^+$ T cells as well as the percentage of $CD4^+$ CD29H cells remained high in the chronic phase in controlled animals (Fig. 7B and 6B, respectively).

Although overall SIV-specific $CD8^+$ T cells were high in Env-vaccinated controlled animals, such correlation was not seen in vector controls grouped as uncontrolled animals (Fig. 7A). Therefore, to examine the relevance of virus-specific T cells to the control of SIV infection, the magnitudes of every viral-protein-specific T cell in controlled and uncontrolled animals were compared. As shown in Fig. 7C, Gag-specific $CD8^+$ T cells and $CD4^+$ T cells, and Tat/Rev-specific $CD4^+$ T cells

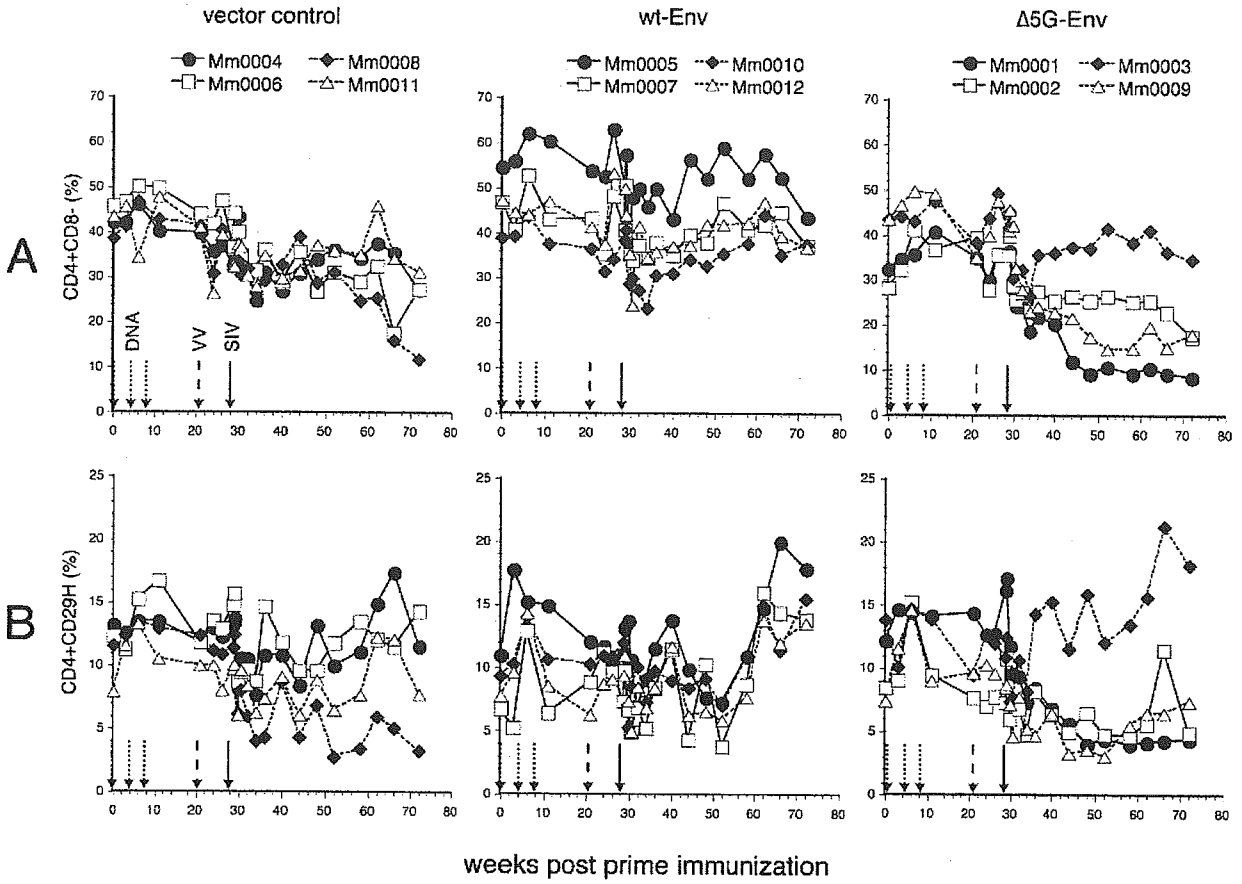


FIG. 6. $CD4^+$ T cells in PBMCs from rhesus macaques during immunization and after the challenge infection. A: Percentage of $CD4^+$ T cells in PBMCs; B: percentage of $CD4^+$ CD29high T cells in PBMCs.

were induced, with statistical significance ($P < 0.05$), in the control animals.

DISCUSSION

The heavily glycosylated structure of Env has been considered a main cause of chronically persistent viral replication and the pathogenicity of HIV/SIV, primarily because it potentially interferes with the development of the host immune response associated with protective immune functions, such as NAb and CTL (10, 36, 44). This characteristic constitutes the primary reason for the difficulty of developing effective vaccines. We therefore examined the efficacy of a deglycosylated-Env vaccine and compared it with the wt-Env vaccine. This study showed that quintuple deglycosylation neither improved the immunogenicity of the wt-Env vaccine nor elicited NAb against SIVmac239. This was in contrast to what occurred with Δ5G infection in rhesus macaques, because the host response elicited by Δ5G infection not only contained Δ5G infection but also protected the animals from SIVmac239 challenge infection (36). This study therefore suggested that an almost sterilizing immunity against SIVmac239 induced in Δ5G-infected animals could not be explained by the immunogenicity of Δ5G Env; instead, it is likely associated with the property of Δ5G as an attenuated virus. In fact, Δ5G was more neutralization-sensitive than SIVmac239 (36). Alternatively, the immunogenic property of Env in Δ5G could not successfully be duplicated by immunization with a Δ5G Env DNA prime-vaccinia virus boost regimen. Therefore, another immunization regimen might be able to elicit the protective immune response induced by Δ5G infection.

The Env vaccine is superior to other vaccines containing other viral proteins with respect to the induction of NAb; however, both the Δ5G Env vaccine and the wt-Env vaccine could not induce detectable NAb against either SIVmac239 or Δ5G. Instead, the wt-Env vaccine induced higher NAb against macrophage-tropic SIV than the Δ5G Env vaccine. Notably, this parameter most significantly correlated with the efficacies of the two Env vaccines. As Ab neutralized the macrophage-tropic variant 239/envMERT, which has only four separate amino acid substitutions distributed in *env* of SVmac239 (34), it might recognize unknown epitopes conserved between SIVmac239 and 239/envMERT. On the other hand, Δ5G Env may not sufficiently present this epitope due to mutations. Regarding the role of nonneutralizing Ab for the control of SIVmac239 infection, it is assumed that, as the neutralization assay did not necessarily reflect in vivo conditions, such nonneutralizing Ab with potential virus-binding ability may interfere with SIVmac239 infection in animals. Alternatively, Ab

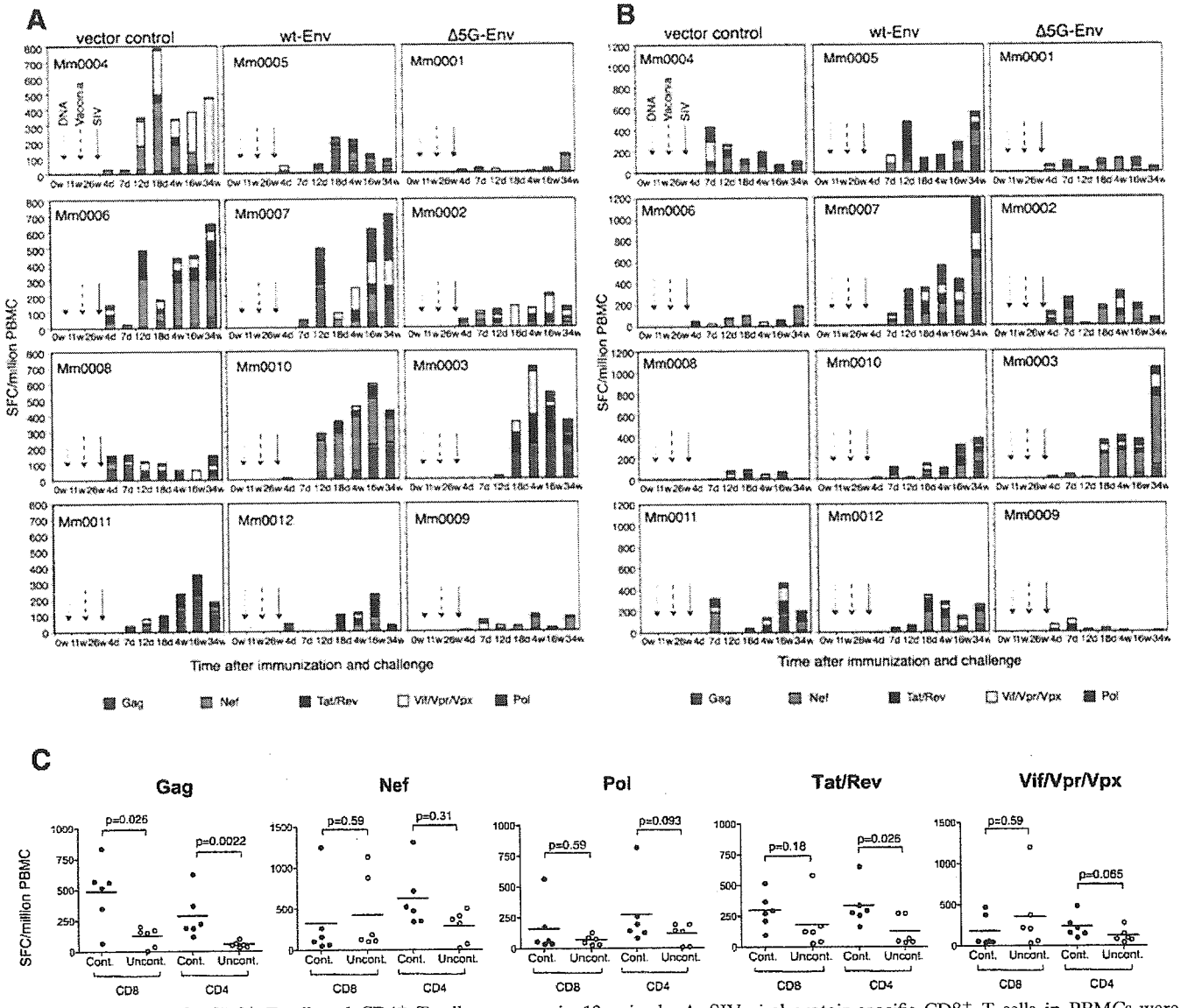


FIG. 7. SIV-specific $CD8^+$ T-cell and $CD4^+$ T-cell responses in 12 animals. A: SIV viral-protein-specific $CD8^+$ T cells in PBMCs were measured by ELISPOT assay for IFN-γ in three groups: vector controls, wt-Env vaccine group, and Δ5G Env vaccines. B: SIV viral-protein-specific $CD4^+$ T cells in PBMCs were measured by ELISPOT assay for IFN-γ in three groups. ELISPOT results of individual SIV proteins are colored as follows: Gag (red), Nef (green), Tat/Rev (blue), Vif/Vpr/Vpx (yellow), and Pol (pink). C: Comparison of cumulated $CD8^+$ T cells or $CD4^+$ T cells specific to the viral proteins Gag, Pol, Nef, Tat/Rev, and Vif/Vpr/VpX between SIV infection-controlled and uncontrolled animals. w, weeks; d, days.

might play a role in other effector functions, such as antibody-dependent cell-mediated cytotoxicity to eliminate the infected cells. The antibody-mediated enhancement of viral antigen processing and cross presentation is also a mechanism potentially related to the control of SIV infection in vivo (49).

Reduced immunogenicity in the Δ5G Env vaccine was also noted in cellular immunity. The levels of stimulation of antigen-specific $CD8^+$ T cells and $CD4^+$ T cells are MHC I and MHC II dependent, respectively. As the macaques in this study have different MHC haplotypes (data not shown), the magnitude and breadth of SIV-specific T cells should vary among the animals. Nevertheless, the magnitude of SU-specific $CD8^+$ T cells and $CD4^+$ T cells in PBMCs was greater in the wt-Env vaccine group than in the Δ5G Env vaccine group. Although the expression of SU by expressing plasmids and that of Env by the vaccinia virus vector elicited by either the wt-Env vaccine or Δ5G Env vaccine were indistinguishable in cultured cells (Fig. 2), wt-Env might persist longer than Δ5G Env in vaccinated animals. T-cell epitopes in the wt-Env vaccine might therefore be more efficiently presented on MHC molecules in antigen-presenting cells than in the Δ5G Env vaccine. Differences in glycosylation levels might also affect some processes in antigen-presenting cells associated with the presentation of T-cell epitopes in Env.

Taking all results together, Env glycosylation might affect the presentation of B-cell epitopes and T-cell epitopes required for Ab-mediated and T-cell-mediated immunities related to the control of SIV infection.

As seen in viral loads and SU-specific T cell levels after challenge infection (Fig. 3 and 5), the effect of vaccination was limited. That seemed related to the development of escape mutants. Therefore, distinctive cellular immune responses after the challenge infection were also implicated in the control of SIVmac239 replication. The magnitude of virus-specific $CD8^+$ T cells did not always correlate with the suppression of viral replication as reported previously (1, 6), particularly in vector controls (Fig. 5 and 7A); however, selected epitope-specific CTL responses might be associated with infection control. Gag-specific CTLs are such candidates, because a high magnitude of Gag-specific $CD8^+$ T cells was significantly elicited in five control animals (Fig. 7C). The magnitude of Gag- or Tat/Rev-specific $CD4^+$ T cells was statistically correlated with infection control (Fig. 7C). This may simply indicate a lower depletion of virus-specific $CD4^+$ T cells in animals with lower viral loads as reported previously (11). Alternatively, these virus-specific $CD4^+$ T cells may play an important role in protective immunity (39). Taken together, these results implicated the dominant role of selected epitope-specific $CD4^+$ T cells and $CD8^+$ T cells for the control of SIVmac239 infection.

The challenge virus that should be used has been an important issue in AIDS vaccine studies (8, 10, 12). Many studies have reported impressive efficacy in a pathogenic-SHIV macaque model (3, 4, 45, 46); however, pathogenic SHIVs use CXCR4 as a coreceptor, whereas the majority of clinical isolates of HIV-1 use CCR5 (13, 27). Therefore, the challenge virus for an AIDS vaccine study should be an R5 virus, such as SIV (10). Consistent with this concern, a DNA prime–modified-vaccinia virus Ankara boost regimen, inducing broad SIV-specific T-cell responses, reduced the initial viral replication but did not prevent disease progression against SIVmac239 challenge (18). Thus, vaccine studies using pathogenic SHIV should be reevaluated by using an R5 virus (10).

Matano et al. reported that a DNA prime-Sendai virus boost regimen induced the CTL-based control of SIVmac239 in rhesus macaques (27). This study demonstrated that a DNA prime-vaccinia virus WR boost regimen expressing only Env controlled the chronic infection of SIVmac239 in rhesus macaques. The relatively lower viral loads in macaques from Myanmar or Laos than in those of Indian origin might contribute to the control of SIVmac239 infection. Nevertheless, it is important that these two studies demonstrated the efficacies of the two vaccine regimens against highly pathogenic SIVmac239. In earlier studies, other R5 SIVs were used as a challenge virus for an efficacy study of vaccine candidates. An Env-based vaccine in vaccinia virus vector priming and subunit protein boosting protected cynomologous macaques against homologous SIVmne clone E11S (42). In recombinant modified vaccinia virus, Ankara viruses expressing Gag-Pol and/or Env exhibited vaccine efficacy because of reduced viremia and the increased survival of rhesus macaques infected with uncloned SIVsmE660 (41). Accordingly, the efficacy of vaccine candidates might be influenced by the experimental conditions. Thus, well-defined animal models with detailed virological, immunological, and genetic information and suitable challenge viruses are required for the evaluation of vaccine candidates and the development of an AIDS vaccine.

This study demonstrated the importance of Env as a component of the AIDS vaccine, and Env-specific $CD8^+$ and $CD4^+$ T cells and nonneutralizing Env-specific Ab were suggested as protective immunity components. Quintuple deglycosylation in Env reduced vaccine efficacy and Env-specific immune responses. Env may therefore be comprised of appropriate antigenic properties to elicit humoral and cellular immune responses required for protective immunity against homologous or allele-specific target SIV/HIV. These properties could be modified by the alteration of glycosylation.

In conclusion, although Env is an important immunogen for the AIDS vaccine, Env properties, including glycosylation, should be carefully considered to design vaccines specific to the targeted viruses.

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HLA-B Polymorphism in Japanese HIV-1–Infected Long-Term Surviving Hemophiliacs

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ABSTRACT

Approximately 30% of patients with hemophilia in Japan were infected with human immunodeficiency virus (HIV) in early 1980s through contaminated blood products. In 1995, a cohort of HIV-infected, asymptomatic patients with hemophilia was set up for follow-up study. Although the patients met the criteria for long-term non-progressor (LTNP) at the entry to the cohort, some of them later developed lymphopenia during five more years of observation. We collected blood samples from 80 long-term survivors; 42 of them did not require antiviral therapy, but the rest were under treatment. Analysis of HLA-B genotype revealed that carriers of known HIV-resistant alleles such as HLA-B*5701, B*5801, and alleles of B27 antigenic group were not increased in frequency, but that HLA-B*1507 was increased in the cohort (6.25% vs. 1.03%, OR = 6.40, $p = 0.039$). We also observed the decrease in carriers of HLA-B*5401 (3.75% vs. 14.95%, OR = 0.22, $p = 0.016$). HLA-B*5401 is a relatively common allele in East Asian populations and belongs to the same B22 antigenic group as B55 and B56 which were reported to associate with rapid progression. Our data indicated that HLA class I is one of the host factors involved in the retardation of HIV disease progression as also reported in the previous studies; however, the alleles associated with this resistance were not the same because of divergent host genetic background.

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INTRODUCTION

Approximately 0.1% of the adult Japanese population is infected with the human immunodeficiency virus (HIV) (1). The number of reported HIV cases, particularly through heterosexual contact, is steadily rising with a doubling of annual cases from the numbers seen in the 1990s to those reported recently (2), but the major source of infection was imported blood products administrated to patients with hemophilia at the beginning of the HIV endemic in Japan (3). The first reported cases of AIDS in Japan were noted in patients with hemophilia in 1985. Thereafter a national HIV/AIDS surveillance study was conducted. By 1998, it was reported that 1404 patients with hemophilia were infected with HIV and that 502 among them had died, after developing AIDS (2). Approximately 30% of hemophiliac patients were found to be HIV carriers within the seven years from 1985 when heated plasma concentrates were generally used. In 1995, we set up a cohort of HIV-infected, asymptomatic patients with hemophilia for follow-up study. Upon the entry to the study, it had already been more than 10 years since exposure to the virus for all patients.

In the natural course of HIV infection, initial viremia accompanied by general manifestations such as fever takes place in the acute phase. Then, most of patients recover more or less from peak viremia to certain levels of viral load or "set points," presumably at least in part through the elimination of viral infected cells by cytotoxic T lymphocyte (CTL) response of the host immune system (4). After years of an asymptomatic period, a considerable number of the patients develop AIDS characterized by the deficit and dysfunction of CD4-positive T lymphocytes along with the re-elevation of viral load, if they do not take any anti-viral measures, such as highly active anti-retroviral treatment (HAART). In the latter viremic phase, the viral genome will have accumulated several mutations, some of which are advantageous to escape from host immune surveillance (4). A small part of infected patients maintain low viral loads, high $CD4^+$ cell count and remain asymptomatic for seven years or more. These are known as long term non-progressors (LTNP) (5). The presence of slow progressors and LTNP has attracted research interests in general because they may hold the underlying biological mechanisms against HIV disease progression.

CTL responses are HLA class I restricted and most immune evasion mutations tend to take place in the coding sequence of the CTL epitope. The CTL epitopes vary with polymorphisms in the peptide binding groove of HLA class I molecule. Therefore HLA class I polymorphism is one candidate factor which elucidates the individual difference in anti-viral response and clinical features. Indeed, there have been many studies which describe that some HLA-alleles are associated with resistance to HIV, and that other alleles were associated with susceptibility (5). In the present study, we examined HLA-B polymorphism in the cohort of Japanese patients with hemophilia who had been infected with HIV for 10 years or longer without progression to AIDS.

MATERIALS AND METHODS

Patient samples. All the protocol of the present study was approved by Ethics Reviewing Board of Yamanashi University and the Medical Research Institute of Tokyo Medical and Dental University as well as all hospitals in which the samples were taken. Upon the set-up of the cohort of HIV-infected Japanese patients with hemophilia in 1995, all patients were infected for longer than ten years but asymptomatic without any antiviral measures. Blood samples were collected from 80 well-characterized patients who were selected from the cohort after obtaining written informed consent. At the time of sample collection, 42 of them were still asymptomatic and maintained their $CD4^+$ T cells at certain level (no less than $200/mm^3$), but the rest were under anti-retroviral treatment (HAART) because of development of CD4 reduction less than $200/mm^3$. Quantification of viral RNA in the plasma was carried out by the Roche Amplicor versions 1.5 assay (Roche Diagnostics, NJ) on the sample collection and will be reported elsewhere in detail. The DNA was prepared from the blood samples by the use of Wizard genomic DNA purification kit (Promega, WI).

HLA-B typing. HLA-B genotype was determined by using Dynal RELI SSO HLA-B typing kit (Dynal Biotech, Oslo, Norway) and Pattern Matching Program (Dynal Biotech) and/or sequence-based typing (SBT) method essentially according to the 13th International HLA Workshop and Congress (IHWC) technical manual published on the IHWC web site ⟨www.ihwc.org⟩. Detailed method of SBT is available on request. In brief, the sequences around exons 2 and 3 were amplified in separate PCR. Amplified DNA was treated with ExoSAP-IT (Amersham Biosciences, NJ) according to the manufacturer's recommendation, and then the sequence was determined by using BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, CA) and ABI 3100 Genetic Analyzer (Applied Biosystems).

Statistical analysis. Frequency of carriers of each allele between patient group and controls were compared. The controls were from random sampling of healthy volunteer ($n = 194$). Odds ratio (OR) was calculated by Woolf's formula (7), for which Haldane's modification was applied upon necessity (8). Statistical significance of the difference in frequencies was evaluated by χ^2 test with Yates' correction (9).