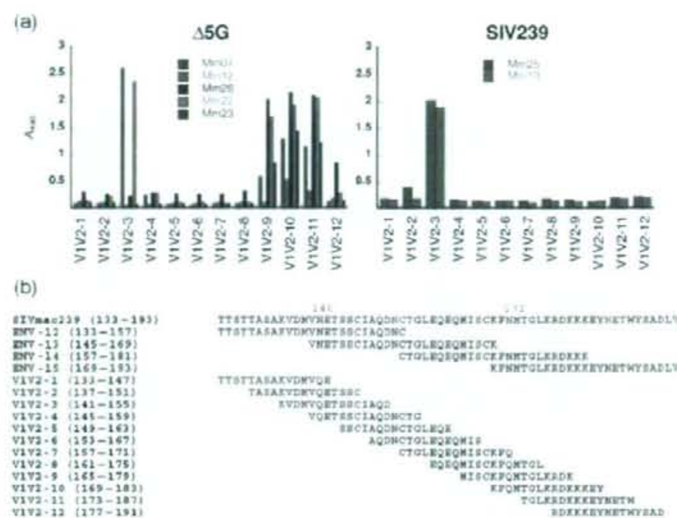

Fig. 5. Ab reactivity to linear epitopes in the V1/V2 region of gp120. (a) To define linear epitopes in the V1/V2 region, peptide ELISA was performed using 12 peptides (15 mers) overlapping by 11 residues each. (b) Sequences and positions of the 12 V1/V2 peptides used in (a) and the peptides Env-12 to -15.

peptide was not detected in any samples, confirming that the epitopes targeted by nAb and V1V2-specific Ab were distinct (data not shown).

Next, we tested plasma IgG samples from SIV-infected animals for the quantitative capture of whole virions. IgG fractions of plasma samples from SIV-infected animals collected at 3–4 weeks p.i. were compared for their capacity to capture Δ5G or SIV239 virions. IgG fractions from two Δ5G-infected animals (Mm07 and Mm22) exhibited remarkably higher virion capture activity than those from other animals (Fig. 7a); however, this capture activity was

Table 1. Epitope-specific Ab-binding regions in Env and influence of deglycosylation on Ab binding

Env subunit	Ab-binding region	Peptide no.	Amino acid range	Region	*P* value*
SU	Region 1	10	109–133	V1	0.6733
		12	133–157	V1	0.5678
		13	145–169	V1/V2	0.5563
		14	157–181	V1/V2	0.0149†
		15	169–193	V1/V2	0.2385
	Region 2	42	493–517	SU C terminus	0.0822
		43	505–529		0.3039
TM	Region 3	50	589–613	Ectodomain	0.4791
		51	601–625		0.0140†
	Region 4	56	660–685	Ectodomain	0.0053‡
	Region 5	61	721–746	Cytoplasmic domain	0.6818
		62	732–757		0.8188
	Region 6	71	841–865	Cytoplasmic domain	0.5237
		72	853–879		0.2451

*A *t*-test was performed by using data in Fig. 4 to determine differences in Ab reactivity between SIV239 infection and Δ5G infection.
†$P<0.05$; ‡$P<0.01$.

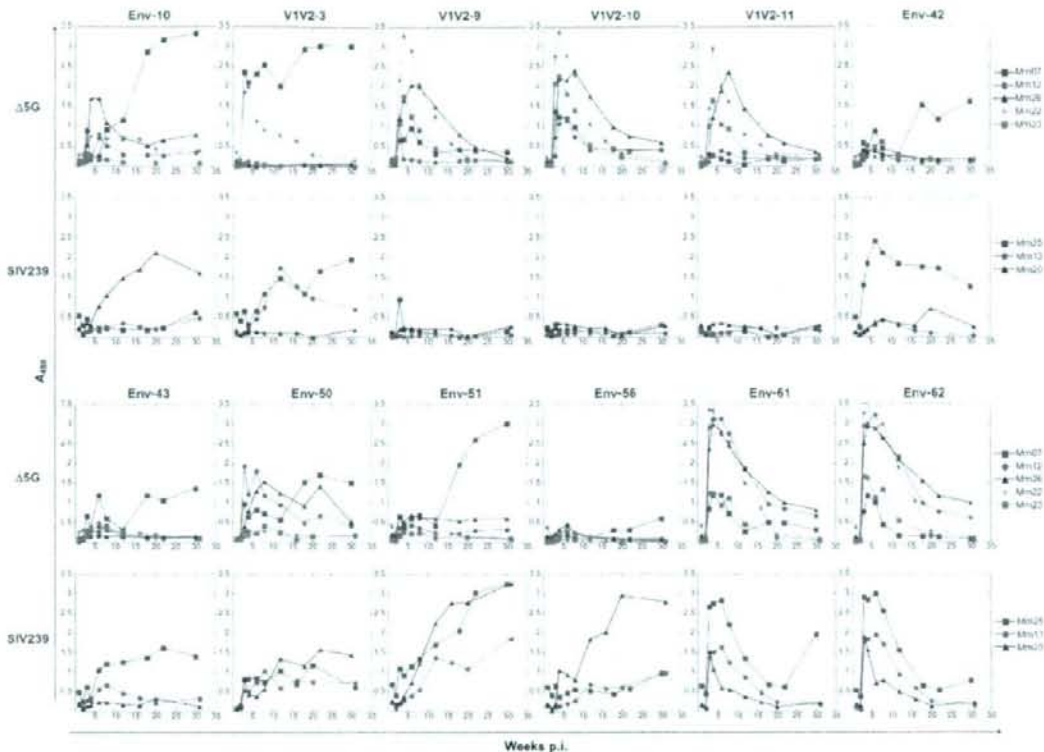

Fig. 6. Kinetics of peptide-specific Ab responses in Δ5G-infected and SIV239-infected animals. The kinetics of Ab reaction against peptides selected in the experiments shown in Figs 4 and 5 was determined as A_{490} using plasma diluted 1 : 100 in an ELISA.

Δ5G-specific, as no appreciable capture of SIV239 virion was detected with these samples. Furthermore, this activity was reduced to the level of control IgG (R374) after selective removal of IgG binding to V1V2-9, -10 and -11 peptides, suggesting that virion capture activity is associated with the Δ5G-specific linear epitope Ab (Fig. 7a). By contrast, IgG fractions from SIV239-infected animals collected at 3–4 weeks p.i. did not exhibit appreciable binding activity either to Δ5G virions or SIV239 virions (Fig. 7b). Thus, these results demonstrated that Δ5G infection elicited not only nAb after 8 weeks p.i., but also a much earlier humoral antiviral mechanism in the form of Δ5G-specific virion-binding Ab at 3–4 weeks p.i. in at least two monkeys (Mm07 and Mm22). To examine the relationship between the two antibody activities, we calculated the correlation of virion capture activity of IgG at 3 or 4 weeks p.i. with a peak nAb titre in Δ5G-infected animals (Fig. 2b) and found that this correlation was statistically significant ($r=1$, $P=0.0167$; Fig. 7c).

DISCUSSION

nAb response in Δ5G-infected animals

Glycosylation of viral spikes has long been recognized as an effective strategy to evade host (humoral) immune surveillance for several pathogens and for HIV/SIV in particular (Dowling *et al.*, 2007; Fournillier *et al.*, 2001; Haigwood & Stamatatos, 2003; Huso *et al.*, 1988; Reitter *et al.*, 1998). In support of these observations, the data presented here demonstrated that quintuple deglycosylation conferred live attenuated vaccine properties to an SIV239 mutant, Δ5G (Mori *et al.*, 2001); however, a cellular but not humoral response was detected as an immune correlate of the protection of Δ5G-infected animals against SIV239 challenge infection. Therefore, we assumed that the complete control of robust acute virus replication in Δ5G-infected animals beyond the initial cell-mediated control would be due to the development of rapid and effective nAbs. This study indicated that, whereas

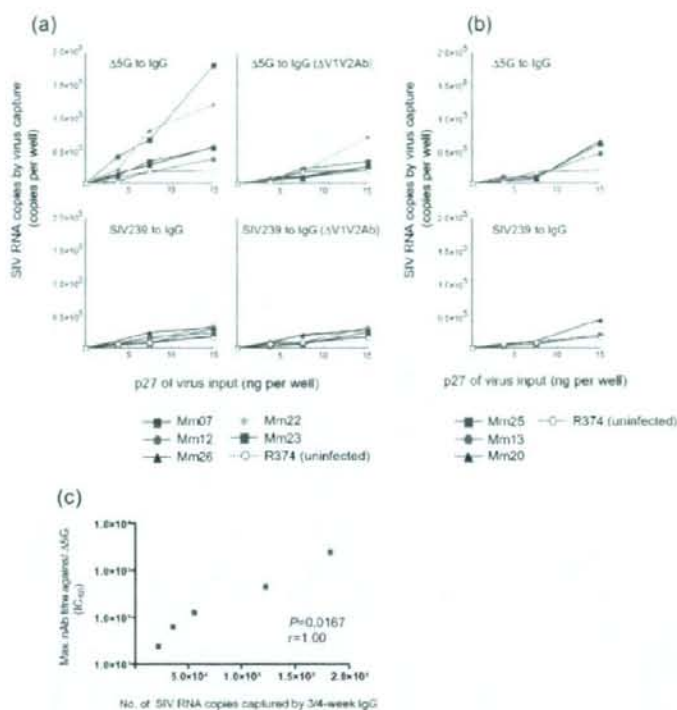

Fig. 7. Virion capture activity of IgG from Δ5G-infected and SIV239-infected animals. Virion capture activity of IgG from the plasma of infected animals at 3 or 4 weeks p.i. was determined by increased captured SIV RNA relative to input (3.75, 7.5 and 15 ng $p27^{gag}$) of Δ5G or SIV239. Plasma samples of Δ5G-infected animals (a) and SIV239-infected animals (b) were used for the assay. IgG (ΔV1V2Ab) indicates IgG depleted of Ab binding to V1V2-9, -10 or -11 peptide. R374 was an uninfected monkey. Correlation between virion capture activity at 3 or 4 weeks p.i. and peak nAb titre in Δ5G-infected animals (Fig. 2b) is shown (c).

Δ5G-infected animals clearly exhibited better nAb responses than SIV239-infected animals, the most stringent nAb assay, based on 90 % inhibition, provided evidence of nAb titres in only two of five Δ5G-infected animals and the appearance of these titres trailed the decline of acute viral loads by almost 4 weeks (Figs 1 and 2). Therefore, we concluded that, although deglycosylation did promote better development of nAbs in Δ5G-infection than SIV239 infection, it was still too late to control acute viraemia.

Zinkernagel and co-workers have categorized viruses into two types: 'acutely cytopathic viruses' and 'poorly or non-cytopathic viruses' (Hangartner *et al.*, 2006b). The former contains viruses such as vesicular stomatitis virus in mice and influenza virus in humans, whose control depends primarily on a rapid and potent nAb response. The latter comprises viruses such as lymphocytic choriomenigitis virus in mice, and hepatitis B and C viruses and HIV in humans, against which a nAb response is apparent only following the reduction of primary viraemia, and which establish persistent chronic infections. Accordingly, although the viral loads in Δ5G infection resembled 'acutely cytopathic virus' infections, the kinetics of nAbs still conformed to the 'non-cytopathic virus' category. As the difference in nAb response between the two types of virus is determined by their surface glycoproteins (Pinschewer *et al.*, 2004), this study suggests that the deglycosylation of Δ5G could not change this intrinsic property of SIV239.

Ab responses to Env peptides in Δ5G-infected animals

Aside from nAb, non-nAb responses to linear epitopes in V1/V2 were specifically induced by 3 weeks p.i. in all Δ5G-infected animals (Figs 4, 5 and 6). The heavy glycosylation of viral spikes clearly prevented access of B-cell receptors to the linear Ab epitopes located within limited regions of gp120 in SIV239, and the reduced glycosylation probably promoted better exposure of these linear epitopes in Δ5G (Fig. 4). Accordingly, the Δ5G-specific epitope in V1/V2 should be closely associated with the deglycosylation mutation at aa 171 in gp120 (Fig. 5). We speculate that this Ab induction might contribute to acute viral suppression in Δ5G infection because of the coincident decrease in peak viraemia (Figs 1 and 6). Non-neutralizing Abs can be divided into those that bind to the intact virion surface and debris-specific Ab. The former non-neutralizing Abs have occasional possibilities for antiviral activities such as antibody-dependent cell-mediated cytotoxicity and complement-mediated virus inactivation (Aasa-Chapman *et al.*, 2005; Ahmad & Menezes, 1996; Forthal *et al.*, 2001; Hangartner *et al.*, 2006a). In fact, readily detectable virion

capture Abs were induced in two of five Δ5G-infected animals (Fig. 7, Mm07 and Mm22). The importance of immediate-early suppression of SIV replication for the long-term containment of infection has been demonstrated by studies of post-exposure anti-retroviral therapy (Lifson *et al.*, 2000; Mori *et al.*, 2000). Thus, the early and complete control of viraemia in Δ5G-infected animals clearly suggests an antiviral mechanism(s) acting as early as 2–4 weeks p.i. Therefore, the early detection of IgG capable of virus capture in Δ5G-infected animals may provide mechanisms capable of contributing to undetectable viral load set points (Fig. 1b). The selective generation of such Ab directed to linear Env epitopes is expected.

Interestingly, deglycosylation in gp120 was also associated with a general reduction in the antigenicity of linear epitopes in gp41: the Ab response against the two epitopes that reside in the regions between the two heptad repeats (aa 601–625) and in the C-terminal heptad repeat (aa 660–685), respectively, was markedly reduced (Fig. 4, Table 1). The former corresponds to the highly conserved immunogenic epitope (Benichou *et al.*, 1993; Gnann *et al.*, 1987; Silvera *et al.*, 1994), and the latter corresponds to an epitope identified in the chronic phase of SIVmac251 infection (Silvera *et al.*, 1994) and corresponds to the nAb epitope of HIV-1 known as 2F5 (Muster *et al.*, 1993), although this linear epitope has not been associated with SIV neutralization (Caffrey *et al.*, 1998). Thus, these epitopes are probably exposed on the surface of viral spikes or their degraded fragments in most SIV and HIV-1 isolates with appropriate glycosylation and correct folding. We believe that the loss of glycosylation might induce a slight conformational change in the gp120 protein backbone, resulting in altered interaction of gp120 and gp41. In fact, the region encompassing the former epitope in gp41 was demonstrated to interact with gp120 (Cao *et al.*, 1993; Maerz *et al.*, 2001; York & Nunberg, 2004). As viral spikes determine virus properties such as viral receptor usage and cell tropism (Kolchinsky *et al.*, 2001; Puffer *et al.*, 2002), different cell populations might be infected in Δ5G-infected animals compared with SIV239 infection. More specifically, because of the distinct properties of the virus, vigorous Δ5G replication in the acute phase did not apparently impair immune function and thus established the control of chronic-phase infection and viral replication.

Host factors required for functional Ab responses against SIV infection

This study also demonstrated remarkable differences in humoral response with regard to nAb and virion capture Ab among Δ5G-infected animals. However, gp120-specific-binding Ab and the linear epitope-specific Ab were initially induced similarly in all animals. These findings imply that Abs measured by ELISA assay and Abs exhibiting antiviral activity are elicited by different pathways and that the properties associated with functional Abs depend largely on the host and underscore the importance of its genetic background. Rhesus macaques are present in various geographical locations within the Asian continent and are subdivided into many subspecies morphologically and genetically (Smith & McDonough, 2005). Some of the genetic differences among rhesus monkeys of different geographical origins, and especially those involving major histocompatibility complex (MHC) genotypes, probably influence the corresponding differences in immune responses, especially cellular response (Bontrop *et al.*, 1996; O'Connor *et al.*, 2003; Reimann *et al.*, 2005). Schmitz *et al.* (2005) reported that Mamu-A*01-positive rhesus monkeys elicited a significantly higher cellular response and lower nAb titres than those in Mamu-A*01-negative animals at the time of challenge infection of animals vaccinated with live attenuated SIV. They suggested that both humoral and cellular immune responses contributed to the protection against the challenge infection and that the relative contribution of each of the responses may be genetically determined. We observed a similar relationship between nAb and cellular responses among Δ5G-infected animals: two animals (Mm07 and Mm22) elicited a lower cellular response while the other three animals (Mm12, Mm23 and Mm26) elicited a higher cellular response (data not shown). Notably two animals exhibiting highly functional Ab (Mm07 and Mm22) were the offspring of seed animals imported from Laos, whilst the others (Mm12, Mm23 and Mm26) were of Burmese origin, suggesting the potential association of such different humoral and cellular responses with host genetic factors. In clinical studies, considerable concordance of adaptive cellular and humoral responses and HIV evolution in monozygotic twins, but not in brothers, infected with the same virus has been reported (Draenert *et al.*, 2006). HIV-1-exposed but uninfected status with significantly higher neutralizing IgA was linked to genotypes on chromosome 22 (Kanari *et al.*, 2005). In the mouse Friend leukemia virus model, MHC II alleles were determined as host genetic factors required for effective nAb response (Miyazawa *et al.*, 1992) and the host genetic factor was mapped to chromosome 15, which was associated with the clearance of viraemia by nAb (Hasenkrug *et al.*, 1995; Kanari *et al.*, 2005).

Taken together, we speculate that the functional humoral response is determined by host genetic properties similar to the cellular immune response. Thus, gaining knowledge of the genetic requirements for both humoral and cellular containment of viral infections will clearly be of primary importance for vaccine development and therapeutics against HIV and other infectious agents.

NOTE ADDED IN PROOF

A discrepancy in the SIV239-infected animals Mm13 and Mm20 was noted between the result shown in Fig. 2 and that in a previous report Mori *et al.*, 2001. The nAb

response against SIV239 in Mm20 was confirmed at multiple time points in the present study.

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REFERENCES

Aasa-Chapman, M. M., Holuigue, S., Aubin, K., Wong, M., Jones, N. A., Cornforth, D., Pellegrino, P., Newton, P., Williams, I. & other authors (2005). Detection of antibody-dependent complement-mediated inactivation of both autologous and heterologous virus in primary human immunodeficiency virus type 1 infection. *J Virol* **79**, 2823–2830.

Ahmad, A. & Menezes, J. (1996). Antibody-dependent cellular cytotoxicity in HIV infections. *FASEB J* **10**, 258–266.

Benichou, S., Venet, A., Beyer, C., Tiollais, P. & Madaule, P. (1993). Characterization of B-cell epitopes in the envelope glycoproteins of simian immunodeficiency virus. *Virology* **194**, 870–874.

Bontrop, R. E., Otting, N., Niphuis, H., Noort, R., Teeuwsen, V. & Heeney, J. L. (1996). The role of major histocompatibility complex polymorphisms on SIV infection in rhesus macaques. *Immunol Lett* **51**, 35–38.

Burns, D. P. & Desrosiers, R. C. (1991). Selection of genetic variants of simian immunodeficiency virus in persistently infected rhesus monkeys. *J Virol* **65**, 1843–1854.

Burton, D. R., Desrosiers, R. C., Doms, R. W., Koff, W. C., Kwong, P. D., Moore, J. P., Nabel, G. J., Sodroski, J., Wilson, I. A. & Wyatt, R. T. (2004). HIV vaccine design and the neutralizing antibody problem. *Nat Immunol* **5**, 233–236.

Caffrey, M., Cai, M., Kaufman, J., Stahl, S. J., Wingfield, P. T., Covell, D. G., Gronenborn, A. M. & Clore, G. M. (1998). Three-dimensional solution structure of the 44 kDa ectodomain of SIV gp41. *EMBO J* **17**, 4572–4584.

Cao, J., Bergeron, L., Helseth, E., Thali, M., Repke, H. & Sodroski, J. (1993). Effects of amino acid changes in the extracellular domain of the human immunodeficiency virus type 1 gp41 envelope glycoprotein. *J Virol* **67**, 2747–2755.

Chackerian, B., Rudensey, L. M. & Overbaugh, J. (1997). Specific *N*-linked and *O*-linked glycosylation modifications in the envelope V1 domain of simian immunodeficiency virus variants that evolve in the host alter recognition by neutralizing antibodies. *J Virol* **71**, 7719–7727.

Chen, B., Vogan, E. M., Gong, H., Skehel, J. J., Wiley, D. C. & Harrison, S. C. (2005). Structure of an unliganded simian immunodeficiency virus gp120 core. *Nature* **433**, 834–841.

Cheng-Mayer, C., Brown, A., Harouse, J., Luciw, P. A. & Mayer, A. J. (1999). Selection for neutralization resistance of the simian/human immunodeficiency virus $SHIV_{SF33A}$ variant in vivo by virtue of sequence changes in the extracellular envelope glycoprotein that modify *N*-linked glycosylation. *J Virol* **73**, 5294–5300.

Choi, W. S., Collignon, C., Thiriart, C., Burns, D. P., Stott, E. J., Kent, K. A. & Desrosiers, R. C. (1994). Effects of natural sequence variation on recognition by monoclonal antibodies neutralize simian immunodeficiency virus infectivity. *J Virol* **68**, 5395–5402.

Dowling, W., Thompson, E., Badger, C., Mellquist, J. L., Garrison, A. R., Smith, J. M., Paragas, J., Hogan, R. J. & Schmaljohn, C. (2007). Influences of glycosylation on antigenicity, immunogenicity, and protective efficacy of Ebola virus GP DNA vaccines. *J Virol* **81**, 1821–1837.

Draenert, R., Allen, T. M., Liu, Y., Wrin, T., Chappey, C., Verrill, C. L., Sirera, G., Eldridge, R. L., Lahaie, M. P. & other authors (2006). Constraints on HIV-1 evolution and immunodominance revealed in monozygotic adult twins infected with the same virus. *J Exp Med* **203**, 529–539.

Eberle, J., Loussert-Ajaka, I., Brust, S., Zekeng, L., Hauser, P. H., Kaptue, L., Knapp, S., Damond, F., Saragosti, S. & other authors (1997). Diversity of the immunodominant epitope of gp41 of HIV-1 subtype O and its validity for antibody detection. *J Virol Methods* **67**, 85–91.

Forthal, D. N., Landucci, G. & Keenan, B. (2001). Relationship between antibody-dependent cellular cytotoxicity, plasma HIV type 1 RNA, and CD4$^+$ lymphocyte count. *AIDS Res Hum Retroviruses* **17**, 553–561.

Fournillier, A., Wychowski, C., Boucreux, D., Baumert, T. F., Meunier, J. C., Jacobs, D., Muguet, S., Depla, E. & Inchauspe, G. (2001). Induction of hepatitis C virus E1 envelope protein-specific immune response can be enhanced by mutation of *N*-glycosylation sites. *J Virol* **75**, 12088–12097.

Gnann, J. W., Jr, Nelson, J. A. & Oldstone, M. B. (1987). Fine mapping of an immunodominant domain in the transmembrane glycoprotein of human immunodeficiency virus. *J Virol* **61**, 2639–2641.

Haigwood, N. L. & Stamatatos, L. (2003). Role of neutralizing antibodies in HIV infection. *AIDS* **17** (Suppl. 4), S67–S71.

Hangartner, L., Zellweger, R. M., Giobbi, M., Weber, J., Eschli, B., McCoy, K. D., Harris, N., Recher, M., Zinkernagel, R. M. & Hengartner, H. (2006a). Nonneutralizing antibodies binding to the surface glycoprotein of lymphocytic choriomeningitis virus reduce early virus spread. *J Exp Med* **203**, 2033–2042.

Hangartner, L., Zinkernagel, R. M. & Hengartner, H. (2006b). Antiviral antibody responses: the two extremes of a wide spectrum. *Nat Rev Immunol* **6**, 231–243.

Hasenkrug, K. J., Valenzuela, A., Letts, V. A., Nishio, J., Chesebro, B. & Frankel, W. N. (1995). Chromosome mapping of *Rfv3*, a host resistance gene to Friend murine retrovirus. *J Virol* **69**, 2617–2620.

Hofmann-Lehmann, R., Swenerton, R. K., Liska, V., Leutenegger, C. M., Lutz, H., McClure, H. M. & Ruprecht, R. M. (2000). Sensitive and robust one-tube real-time reverse transcriptase-polymerase chain reaction to quantify SIV RNA load: comparison of one- versus two-enzyme systems. *AIDS Res Hum Retroviruses* **16**, 1247–1257.

Huso, D. L., Narayan, O. & Hart, G. W. (1988). Sialic acids on the surface of caprine arthritis-encephalitis virus define the biological properties of the virus. *J Virol* **62**, 1974–1980.

Johnson, W. E., Sanford, H., Schwall, L., Burton, D. R., Parren, P. W., Robinson, J. E. & Desrosiers, R. C. (2003). Assorted mutations in the envelope gene of simian immunodeficiency virus lead to loss of neutralization resistance against antibodies representing a broad spectrum of specificities. *J Virol* **77**, 9993–10003.

Kanari, Y., Clerici, M., Abe, H., Kawabata, H., Trabattoni, D., Caputo, S. L., Mazzotta, F., Fujisawa, H., Niwa, A. & other authors (2005). Genotypes at chromosome 22q12–13 are associated with HIV-1-exposed but uninfected status in Italians. *AIDS* **19**, 1015–1024.

Kent, K. A., Rud, E., Corcoran, T., Powell, C., Thiriart, C., Collignon, C. & Stott, E. J. (1992). Identification of two neutralizing and 8 non-neutralizing epitopes on simian immunodeficiency virus envelope

using monoclonal antibodies. *AIDS Res Hum Retroviruses* **8**, 1147–1151.

Kolchinsky, P., Kiprilov, E., Bartley, P., Rubinstein, R. & Sodroski, J. (2001). Loss of a single *N*-linked glycan allows CD4-independent human immunodeficiency virus type 1 infection by altering the position of the gp120 V1/V2 variable loops. *J Virol* **75**, 3435–3443.

Leonard, C. K., Spellman, M. W., Riddle, L., Harris, R. J., Thomas, J. N. & Gregory, T. J. (1990). Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. *J Biol Chem* **265**, 10373–10382.

Lifson, J. D., Rossio, J. L., Arnaout, R., Li, L., Parks, T. L., Schneider, D. K., Kiser, R. F., Coalter, V. J., Walsh, G. & other authors (2000). Containment of simian immunodeficiency virus infection: cellular immune responses and protection from rechallenge following transient postinoculation antiretroviral treatment. *J Virol* **74**, 2584–2593.

Liu, J., Wang, S., Hoxie, J. A., LaBranche, C. C. & Lu, M. (2002). Mutations that destabilize the gp41 core are determinants for stabilizing the simian immunodeficiency virus-CPmac envelope glycoprotein complex. *J Biol Chem* **277**, 12891–12900.

Maerz, A. L., Drummer, H. E., Wilson, K. A. & Poumbourios, P. (2001). Functional analysis of the disulfide-bonded loop/chain reversal region of human immunodeficiency virus type 1 gp41 reveals a critical role in gp120-gp41 association. *J Virol* **75**, 6635–6644.

Means, R. E., Greenough, T. & Desrosiers, R. C. (1997). Neutralization sensitivity of cell culture-passaged simian immunodeficiency virus. *J Virol* **71**, 7895–7902.

Miyazawa, M., Nishio, J., Wehrly, K. & Chesebro, B. (1992). Influence of MHC genes on spontaneous recovery from Friend retrovirus-induced leukemia. *J Immunol* **148**, 644–647.

Mori, K., Yasutomi, Y., Sawada, S., Villinger, F., Sugama, K., Rosenwith, B., Heeney, J. L., Uberla, K., Yamazaki, S. & other authors (2000). Suppression of acute viremia by short-term postexposure prophylaxis of simian/human immunodeficiency virus SHIV-RT-infected monkeys with a novel reverse transcriptase inhibitor (GW420867) allows for development of potent antiviral immune responses resulting in efficient containment of infection. *J Virol* **74**, 5747–5753.

Mori, K., Yasutomi, Y., Ohgimoto, S., Nakasone, T., Takamura, S., Shioda, T. & Nagai, Y. (2001). Quintuple deglycosylation mutant of simian immunodeficiency virus SIVmac239 in rhesus macaques: robust primary replication, tightly contained chronic infection, and elicitation of potent immunity against the parental wild-type strain. *J Virol* **75**, 4023–4028.

Mori, K., Sugimoto, C., Ohgimoto, S., Nakayama, E. E., Shioda, T., Kusagawa, S., Takebe, Y., Kano, M., Matano, T. & other authors (2005). Influence of glycosylation on the efficacy of an Env-based vaccine against simian immunodeficiency virus SIVmac239 in a macaque AIDS model. *J Virol* **79**, 10386–10396.

Muster, T., Steindl, F., Purtscher, M., Trkola, A., Klima, A., Himmler, G., Ruker, F. & Katinger, H. (1993). A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. *J Virol* **67**, 6642–6647.

Nishimura, Y., Igarashi, T., Haigwood, N., Sadjadpour, R., Plishka, R. J., Buckler-White, A., Shibata, R. & Martin, M. A. (2002). Determination of a statistically valid neutralization titer in plasma that confers protection against simian-human immunodeficiency virus challenge following passive transfer of high-titered neutralizing antibodies. *J Virol* **76**, 2123–2130.

Nyambi, P. N., Gorny, M. K., Bastiani, L., van der Groen, G., Williams, C. & Zolla-Pazner, S. (1998). Mapping of epitopes exposed on intact human immunodeficiency virus type 1 (HIV-1) virions: a new strategy for studying the immunologic relatedness of HIV-1. *J Virol* **72**, 9384–9391.

O'Connor, D. H., Mothe, B. R., Weinfurter, J. T., Fuenger, S., Rehrauer, W. M., Jing, P., Rudersdorf, R. R., Liebl, M. E., Krebs, K. & other authors (2003). Major histocompatibility complex class I alleles associated with slow simian immunodeficiency virus disease progression bind epitopes recognized by dominant acute-phase cytotoxic-T-lymphocyte responses. *J Virol* **77**, 9029–9040.

Ohgimoto, S., Shioda, T., Mori, K., Nakayama, E. E., Hu, H. & Nagai, Y. (1998). Location-specific, unequal contribution of the *N* glycans in simian immunodeficiency virus gp120 to viral infectivity and removal of multiple glycans without disturbing infectivity. *J Virol* **72**, 8365–8370.

Pinschewer, D. D., Perez, M., Jeetendra, E., Bachi, T., Horvath, E., Hengartner, H., Whitt, M. A., de la Torre, J. C. & Zinkernagel, R. M. (2004). Kinetics of protective antibodies are determined by the viral surface antigen. *J Clin Invest* **114**, 988–993.

Puffer, B. A., Pohlmann, S., Edinger, A. L., Carlin, D., Sanchez, M. D., Reitter, J., Watry, D. D., Fox, H. S., Desrosiers, R. C. & Doms, R. W. (2002). CD4 independence of simian immunodeficiency virus Envs is associated with macrophage tropism, neutralization sensitivity, and attenuated pathogenicity. *J Virol* **76**, 2595–2605.

Regier, D. A. & Desrosiers, R. C. (1990). The complete nucleotide sequence of a pathogenic molecular clone of simian immunodeficiency virus. *AIDS Res Hum Retroviruses* **6**, 1221–1231.

Reimann, K. A., Parker, R. A., Seaman, M. S., Beaudry, K., Beddall, M., Peterson, L., Williams, K. C., Veazey, R. S., Montefiori, D. C. & other authors (2005). Pathogenicity of simian-human immunodeficiency virus SHIV-89.6P and SIVmac is attenuated in cynomolgus macaques and associated with early T-lymphocyte responses. *J Virol* **79**, 8878–8885.

Reitter, J. N., Means, R. E. & Desrosiers, R. C. (1998). A role for carbohydrates in immune evasion in AIDS. *Nat Med* **4**, 679–684.

Schmitz, J. E., Johnson, R. P., McClure, H. M., Manson, K. H., Wyand, M. S., Kuroda, M. J., Lifton, M. A., Khunkhun, R. S., McEvers, K. J. & other authors (2005). Effect of $CD8^+$ lymphocyte depletion on virus containment after simian immunodeficiency virus SIVmac251 challenge of live attenuated SIVmac239Δ3-vaccinated rhesus macaques. *J Virol* **79**, 8131–8141.

Silvera, P., Flanagan, B., Kent, K., Rud, E., Powell, C., Corcoran, T., Bruck, C., Thiriart, C., Haigwood, N. L. & Stott, E. J. (1994). Fine analysis of humoral antibody response to envelope glycoprotein of SIV in infected and vaccinated macaques. *AIDS Res Hum Retroviruses* **10**, 1295–1304.

Smith, D. G. & McDonough, J. (2005). Mitochondrial DNA variation in Chinese and Indian rhesus macaques (*Macaca mulatta*). *Am J Primatol* **65**, 1–25.

Wei, X., Decker, J. M., Wang, S., Hui, H., Kappes, J. C., Wu, X., Salazar-Gonzalez, J. F., Salazar, M. G., Kilby, J. M. & other authors (2003). Antibody neutralization and escape by HIV-1. *Nature* **422**, 307–312.

Wyatt, R. & Sodroski, J. (1998). The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. *Science* **280**, 1884–1888.

Wyatt, R., Kwong, P. D., Desjardins, E., Sweet, R. W., Robinson, J., Hendrickson, W. A. & Sodroski, J. G. (1998). The antigenic structure of the HIV gp120 envelope glycoprotein. *Nature* **393**, 705–711.

York, J. & Nunberg, J. H. (2004). Role of hydrophobic residues in the central ectodomain of gp41 in maintaining the association between human immunodeficiency virus type 1 envelope glycoprotein subunits gp120 and gp41. *J Virol* **78**, 4921–4926.

Yu, D., Shioda, T., Kato, A., Hasan, M. K., Sakai, Y. & Nagai, Y. (1997). Sendai virus-based expression of HIV-1 gp120: reinforcement by the V(−) version. *Genes Cells* **2**, 457–466.

Zhang, M., Gaschen, B., Blay, W., Foley, B., Haigwood, N., Kuiken, C. & Korber, B. (2004). Tracking global patterns of *N*-linked glycosylation site variation in highly variable viral glycoproteins: HIV, SIV, and HCV envelopes and influenza hemagglutinin. *Glycobiology* **14**, 1229–1246.

Zolla-Pazner, S. (2004). Identifying epitopes of HIV-1 that induce protective antibodies. *Nat Rev Immunol* **4**, 199–210.

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Antigen-specific T-cell induction by vaccination with a recombinant Sendai virus vector even in the presence of vector-specific neutralizing antibodies in rhesus macaques

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ABSTRACT

Recombinant viral vectors are promising vaccine tools for eliciting potent cellular immune responses against immunodeficiency virus infection, but pre-existing anti-vector antibodies can be an obstacle to their clinical use in humans. We have previously vaccinated rhesus macaques with a recombinant Sendai virus (SeV) vector twice at an interval of more than 1 year and have shown efficient antigen-specific T-cell induction by the second as well as the first vaccination. Here, we have established the method for measurement of SeV-specific neutralizing titers and have found efficient SeV-specific neutralizing antibody responses just before the second SeV vaccination in these macaques. This suggests the feasibility of inducing antigen-specific T-cell responses by SeV vaccination even in the host with pre-existing anti-SeV neutralizing antibodies.

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Virus-specific CD8+ cytotoxic T lymphocyte (CTL) responses are crucial for the control of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) replication [1–5]. Efficient CTL induction is a key strategy for AIDS vaccine development, and recombinant viral vectors are promising tools for its elicitation [6]. Most of the parental or related viruses of these vectors can induce natural infection in humans. Thus, pre-existing immunity against the vector virus itself could be an obstacle to viral vector-based efficient CTL induction in humans. Indeed, a clinical trial of a vaccine using adenovirus serotype 5 (AdV5) vectors has shown reduction in efficiency of vaccine-based CTL induction in people with pre-existing anti-AdV5 antibodies [7–9].

We have developed an AIDS vaccine using a recombinant Sendai virus (SeV) vector and have shown its potential for efficient CTL induction in macaques [10–12]. SeV, murine parainfluenza virus type 1 (PIV-1), is an enveloped virus with a negative-sense RNA genome. Its natural host is mice and its natural infection is not believed to occur efficiently in primates including humans [11,13]. However, antibodies against human PIV-1 (hPIV-1), whose natural infection frequently occurs in humans, are known to cross-react with SeV [14]. It can be expected that the presence of these cross-reactive antibodies at SeV vaccination may reduce its efficiency of CTL induction, but it remains unclear how much extent anti-hPIV-1 antibodies may have adverse effect on SeV-based CTL induction in humans.

Recently, we have vaccinated four rhesus macaques with SeV vectors twice at an interval of more than 1 year and examined antigen-specific CTL induction by the second SeV vaccination [15]. The second vaccination of macaques with an SIV Gag-expressing SeV (SeV-Gag) vector resulted in efficient induction of Gag-specific CTL responses. In the present study, we have established the method for measurement of SeV-specific neutralizing titers and examined SeV-specific neutralizing antibody responses at the second SeV-Gag vaccination in these four macaques. Our results revealed that Gag-specific CTL responses were induced by the second SeV-Gag vaccination in the presence of anti-SeV neutralizing antibodies.

Materials and methods

Samples. Plasma samples were obtained from four Burmese rhesus macaques (*Macaca mulatta*), R011, R012, R003, and R006, that received SeV vaccination twice as described previously [15]. In brief, macaques R011 and R012 received four times of vaccinations with an env- and nef-deleted simian–human immunodeficiency virus (SHIV) molecular clone DNA [16] and a single intranasal boost with a replication-competent SeV-Gag (F[+]SeV-

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Gag) [10,17], whereas macaques R003 and R006 were boosted intranasally with a recombinant SeV expressing HIV-1 Tat (F[+]SeV-Tat) after the DNA vaccinations [18]. Animals were challenged intravenously with SHIV89.6PD approximately 3 months after the SeV boost. Finally, these macaques received the second SeV vaccination; macaques R011 and R012 were vaccinated with replication-defective F-deleted SeV-Gag (F^{−}SeV-Gag) [19] at week 191 and euthanized at week 196 after the first F(+)SeV-Gag vaccination, whereas R003 and R006 were vaccinated with F(+)SeV-Gag at week 68 and euthanized at week 69 (R003) or 70 (R006) after the first F(+)SeV-Tat vaccination. These macaques were maintained in accordance with the Guideline for Laboratory animals of National Institute of Infectious Diseases and National Institute of Biomedical Innovation.

Measurement of plasma anti-SeV IgG levels. The plasma anti-SeV immunoglobulin G (IgG) levels were measured by an enzyme-linked immunosorbent assay (ELISA) (Denka Seiken, Tokyo, Japan) using whole inactivated SeV (HVJ Z strain) particles and a peroxidase-conjugated anti-monkey IgG antibody [20].

SeV neutralization assay. A recombinant SeV expressing EGFP (SeV-EGFP) was obtained as described before [17]. Virus titer was determined by infecting LLCMK2 cells and counting the number

Table 1
SeV vaccination and Gag-specific $CD8^+$ T-cell responses

Macaques	SeV vaccination[a]		Gag-specific CD8 T-cell frequencies[b]	
	1st	2nd	Just before the 2nd SeV	1 week after the 2nd SeV
	At week 0	At week 191	At week 191	At week 192
R011	F(+)SeV-Gag	F(−)SeV-Gag	5.0×10^2	1.1×10^3
R012	F(+)SeV-Gag	F(−)SeV-Gag	1.6×10^2	1.3×10^3
	At week 0	At week 68	At week 68	At week 69
R003	F(+)SeV-Tat	F(+)SeV-Gag	5.0×10^2	2.2×10^3
R006	F(+)SeV-Tat	F(+)SeV-Gag	2.5×10^2	1.2×10^3

[a] Macaques R011 and R012 received the second SeV vaccination at week 191 after the first SeV vaccination, and R003 and R006 at week 68.
[b] Gag-specific $CD8^+$ T-cell frequencies per million peripheral blood mononuclear cells (PBMCs) reported previously [15] are shown.

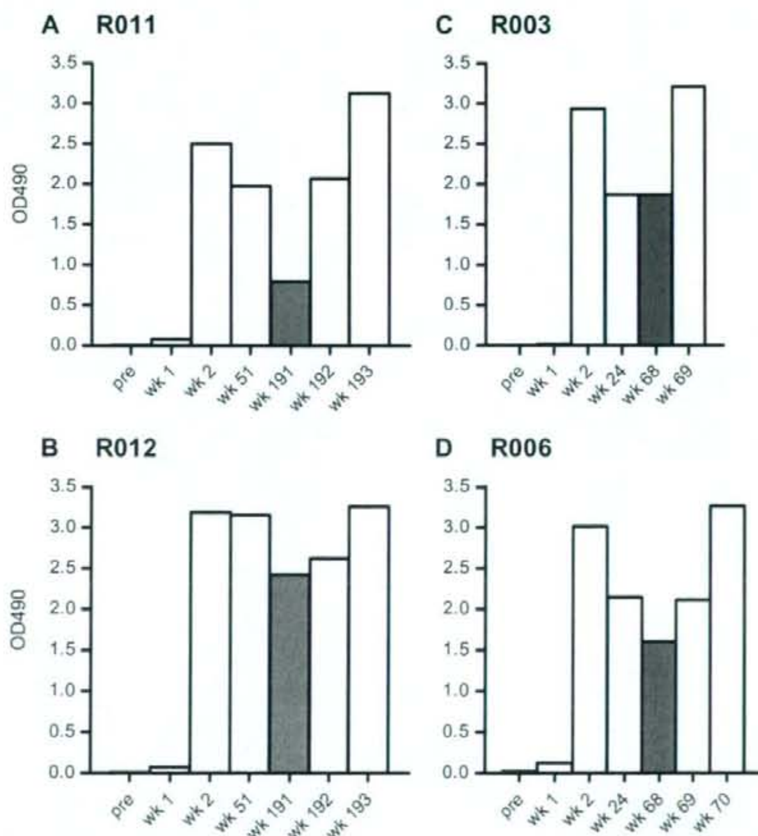

Fig. 1. SeV-specific IgG levels in plasma. Plasma samples obtained from macaques R011 (A), R012 (B), R003 (C), and R006 (D) before the initial DNA vaccination (pre) and at several time points after the first SeV vaccination were diluted by 1/5000 and subjected to ELISA assay. OD490, optical density at 490 nm.

of GFP-expressing cells. To assess the infectivity of SeV-EGFP on LLCMK2 cells, cells were plated at a density of 3.0×10^4 cells per well in 96-well plates, incubated overnight, and infected with serial twofold dilutions of SeV-EGFP. One day after the infection, cells were harvested by using 0.05% trypsin with 0.02% EDTA and subjected to flow-cytometric analysis for detection of EGFP-positive cells.

To measure SeV-specific neutralizing titers on LLCMK2 cells, cells were plated at a density of 3.0×10^4 cells per well in 96-well plates, incubated overnight, and infected with the mixture of SeV-EGFP and diluted plasma. For preparation of the mixture, 25 μl of virus solutions containing 8.3×10^4 cell infectious units (CIU) of SeV-EGFP were incubated with equal volume of serial twofold dilutions of heat-inactivated plasma samples at 37 °C for 1 h. One day after the infection, cells were harvested and subjected to flow-cytometric analysis for detection of EGFP-positive cells. Percent neutralizing activity was calculated by subtracting the percentage of the EGFP-positive cell number in the culture with plasma samples per that without plasma from 100%.

Results and discussion

In the present study, we examined SeV-specific antibody responses in plasma samples of four rhesus macaques that had received SeV vector vaccination twice as described previously [15]. At the second vaccination, macaques R011 and R012 received F(−)SeV-Gag at week 191 after the first vaccination, while macaques R003 and R006 received F(+)SeV-Gag at week 68. In all these macaques, Gag-specific CD8+ T-cell responses were augmented after the second vaccination (Table 1).

We first measured plasma anti-SeV IgG levels in these macaques (Fig. 1). All the macaques showed efficient induction of SeV-specific antibody responses 2 weeks after the first SeV vaccination. High levels of anti-SeV IgG were maintained until the second vaccination and enhancement of the SeV-specific antibody responses were observed after that. This enhancement appeared 1 week after the second SeV vaccination, indicating rapid secondary responses.

Next, we established a method for measurement of SeV-specific neutralizing titers on LLCMK2 cells by using SeV-EGFP. Titration of SeV-EGFP on LLCMK2 cells roughly exhibited a proportional relationship between viral titers and GFP-positive cell frequencies in the m.o.i. (multiplicity of infection) range from 0.2 (6.3×10^3 CIU/well) to 3.3 (1.0×10^5 CIU/well) (Fig. 1A). Then, in the neutralizing assay, 25 μl of virus solution containing 8.3×10^4 CIU of SeV-EGFP was mixed with 25 μl of diluted plasma and added into each well (m.o.i. = 2.8), and GFP-positive cell frequencies were measured.

Incubation of SeV-EGFP with serially diluted pre-immune plasma samples did not affect SeV infectivity and showed similar levels (approximately 75%) of GFP-positive cell frequencies (Fig. 2B). In contrast, neutralization of SeV infection was observed by incubation with plasma samples obtained at week 2 after the first SeV vaccination (Fig. 2B).

We then measured SeV-specific neutralizing titers just before the second SeV vaccination. We determined the end-point plasma titers required for 10-fold reduction of GFP-positive cell frequencies compared to the negative control without plasma (90% neutralization) (Fig. 3). Analyses revealed efficient SeV-specific neutralizing titers in plasma just before the second SeV vaccination in all four macaques. The 90% neutralization titers were 1:100 in macaques R003 and R006, 1:200 in macaque R011, and 1:800 in macaque R012. Higher neutralizing titer in macaque R012 compared to other three was compatible with their anti-SeV IgG levels. The second SeV vaccination resulted in increases in SeV-specific neutralizing titers by fourfold or more.

In macaques R011 and R012, Gag-specific CD8+ T-cell frequencies (cells/million PBMCs) after the first SeV-Gag vaccination were

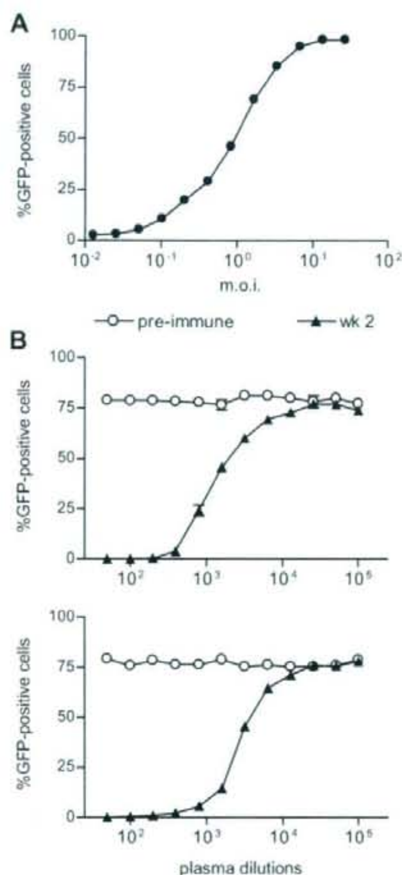

Fig. 2. GFP-positive cell frequencies after SeV-EGFP infection. (A) GFP-positive cell frequencies after infection with diluted SeV-EGFP. LLCMK2 cells were infected with serial twofold dilutions of SeV-EGFP. In case of m.o.i. of 3.3, 3.0×10^4 cells were plated per well in the 96-well plate, incubated overnight, and infected with 1.0×10^5 CIU of SeV-EGFP. The mean values obtained by duplicate experiments are shown. (B) GFP-positive cell frequencies after infection with the mixture of SeV-EGFP and diluted plasma samples. SeV-EGFP (8.3×10^4 CIU) was incubated with serially diluted plasma obtained pre-vaccination (pre-immune, open circles) or at week 2 after the first SeV immunization (closed triangles) from macaques R011 (upper panel) and R003 (lower panel) and added into LLCMK2 cells plated at 3.0×10^4 cells per well. The mean values obtained by duplicate experiments are shown.

approximately 1.0×10^3 [10] and those after the second SeV-Gag vaccination were just above 1.0×10^3 (Table 1) [15]. Granted that the priming conditions and the utilized vectors were different between the first and the second SeV vaccination, these results indicate efficient Gag-specific CD8+ T-cell induction even by the second SeV-Gag vaccination. Thus, our finding of SeV-specific neutralizing antibody responses just before the second SeV-Gag vaccination in the present study indicates the potential of recombinant SeV vectors to induce antigen-specific T-cell responses even in the presence of SeV-specific neutralizing antibodies, suggesting an important implication for development of an effective AIDS vaccine using viral vectors.

Because of the cross-reactivity of anti-SeV antibodies with hPIV-1, a clinical trial phase I of wild-type SeV vaccination against hPIV-1 infection has been performed [21]. However, the potential

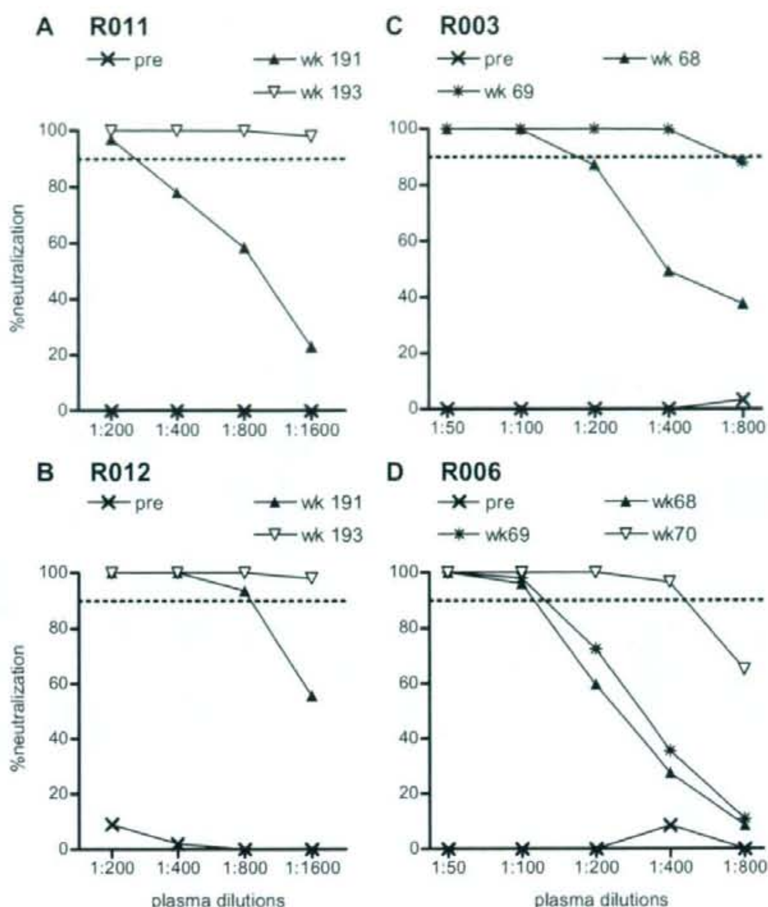

Fig. 3. SeV-specific neutralizing titers in plasma. We examined neutralizing titers in plasma samples obtained before the initial DNA vaccination (pre), just before the second SeV vaccination (at week 191 in R011 and R012 and at week 68 in R003 and R006), and after that in macaques R011 (A), R012 (B), R003 (C), and R006 (D). The mean values obtained by duplicate experiments are shown.

of SeV vaccination to induce hPIV-1-specific neutralizing antibody responses has not precisely evaluated. Conversely, anti-hPIV-1 antibodies may cross-react with SeVs, but how much extent these can neutralize SeVs remains unclear. While we do not know the frequency of anti-hPIV-1 antibody-positive individuals, the present study strongly suggests the feasibility of efficiently inducing antigen-specific T-cell responses by SeV vaccination even in the host with pre-existing anti-hPIV-1 antibodies. Precise evaluation of relationship between pre-existing SeV-specific neutralizing titers and efficiency of antigen-specific T-cell induction would contribute to estimation of SeV vector vaccine efficacy in anti-hPIV-1 antibody-positive individuals.

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References

[1] R.A. Koup, J.T. Safrit, Y. Cao, C.A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, D.D. Ho, Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome, J. Virol. 68 (1994) 4650–4655.

[2] P. Borrow, H. Lewicki, B.H. Hahn, G.M. Shaw, M.B. Oldstone, Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection, J. Virol. 68 (1994) 6103–6110.

[3] T. Matano, R. Shibata, C. Siemon, M. Connors, H.C. Lane, M.A. Martin, Administration of an anti-CD8 monoclonal antibody interferes with the clearance of chimeric simian/human immunodeficiency virus during primary infections of rhesus macaques, J. Virol. 72 (1998) 164–169.

[4] X. Jin, D.E. Bauer, S.E. Tuttleton, S. Lewin, A. Gettie, J. Blanchard, C.E. Irwin, J.T. Safrit, J. Mittler, L. Weinberger, L.G. Kostrikis, L. Zhang, A.S. Perelson, D.D. Ho, Dramatic rise in plasma viremia after CD8(+) T-cell depletion in simian immunodeficiency virus-infected macaques, J. Exp. Med. 189 (1999) 991–998.

[5] J.E. Schmitz, M.J. Kuroda, S. Santra, V.G. Sasseville, M.A. Simon, M.A. Lifton, P. Racz, K. Tenner-Racz, M. Dalesandro, B.J. Scallon, J. Ghrayeb, M.A. Forman, D.C. Montefiori, E.P. Rieber, N.L. Letvin, K.A. Reimann, Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes, Science 283 (1999) 857–860.

[6] A.J. McMichael, T. Hanke, HIV vaccines 1983–2003, Nat. Med. 9 (2003) 874–880.

[7] S.M. Sumida, D.M. Truitt, A.A.C. Lemckert, R. Vogels, J.H.H.V. Custers, M.M. Addo, S. Lockman, T. Peter, F.W. Peyerl, M.G. Kishko, S.S. Jackson, D.A. Gorgone, M.A. Lifton, M. Essex, B.D. Walker, J. Goudsmit, M.J.E. Havenga, D.H. Barouch, Neutralizing antibodies to adenovirus serotype 5 vaccine vectors are directed primarily against the adenovirus hexon protein, J. Immunol. 174 (2005) 7179–7185.

[8] A.T. Catanzaro, R.A. Koup, M. Roederer, R.T. Bailer, M.E. Enama, Z. Moodie, L. Gu, J.E. Martin, L. Novik, B.K. Chakrabarti, B.T. Butman, J.G. Gall, C.R. King, C.A. Andrews, R. Sheets, P.L. Gomez, J.R. Mascola, G.J. Nabel, B.S. Graham, Phase 1 safety and immunogenicity evaluation of a multiclade HIV-1 candidate vaccine delivered by a replication-defective recombinant adenovirus vector, J. Infect. Dis. 194 (2006) 1638–1649.

[9] F.H. Priddy, D. Brown, J. Kublin, K. Monahan, D.P. Wright, J. Lalezari, S. Santiago, M. Marmor, M. Lally, R.M. Novak, S.J. Brown, P. Kulkarni, S.A. Dubey, L.S. Kierstead, D.R. Casimiro, R. Mogg, M.J. Dinubile, J.W. Shiver, R.Y. Leavitt, M.N. Robertson, D.V. Mehrotra, E. Quirk, Safety and immunogenicity of a replication-incompetent adenovirus type 5 HIV-1 clade B gag/pol/nef vaccine in healthy adults, Clin. Infect. Dis (2008). Epub ahead of print.

[10] T. Matano, M. Kano, H. Nakamura, A. Takeda, Y. Nagai, Rapid appearance of secondary immune responses and protection from acute CD4 depletion after a highly pathogenic immunodeficiency virus challenge in macaques vaccinated with a DNA prime/Sendai virus vector boost regimen, J. Virol. 75 (2001) 11891–11896.

[11] M. Kano, T. Matano, A. Kato, H. Nakamura, A. Takeda, Y. Suzaki, Y. Ami, K. Terao, Y. Nagai, Primary replication of a recombinant Sendai virus vector in macaques, J. Gen. Virol. 83 (2002) 1377–1386.

[12] T. Matano, M. Kobayashi, H. Igarashi, A. Takeda, H. Nakamura, M. Kano, C. Sugimoto, K. Mori, A. Iida, T. Hirata, M. Hasegawa, T. Yuasa, M. Miyazawa, Y. Takahashi, M. Yasunami, A. Kimura, D.H. O'Connor, D.I. Watkins, Y. Nagai, Cytotoxic T lymphocyte-based control of simian immunodeficiency virus replication in a preclinical AIDS vaccine trial, J. Exp. Med. 199 (2004) 1709–1718.

[13] Y. Nagai, Paramyxovirus replication and pathogenesis. Reverse genetics transforms understanding, Rev. Med. Virol. 9 (1999) 83–99.

[14] M.H. Skiadopoulos, S.R. Surman, J.M. Riggs, W.R. Elkins, M. St Claire, M. Nishio, D. Garcin, D. Kolakofsky, P.L. Collins, B.R. Murphy, Sendai virus, a murine parainfluenza virus type 1, replicates to a level similar to human PIV1 in the upper and lower respiratory tract of African green monkeys and chimpanzees, Virology 297 (2002) 153–160.

[15] M. Kato, H. Igarashi, A. Takeda, Y. Sasaki, H. Nakamura, M. Kano, T. Sata, A. Iida, M. Hasegawa, S. Horie, E. Higashihara, Y. Nagai, T. Matano, Induction of Gag-specific T-cell responses by therapeutic immunization with a Gag-expressing Sendai virus vector in macaques chronically infected with simian-human immunodeficiency virus, Vaccine 23 (2005) 3166–3173.

[16] T. Matano, M. Kano, T. Odawara, H. Nakamura, A. Takeda, K. Mori, T. Sato, Y. Nagai, Induction of protective immunity against pathogenic simian immunodeficiency virus by a foreign receptor-dependent replication of an engineered avirulent virus, Vaccine 18 (2000) 3310–3318.

[17] A. Kato, K. Kiyotani, Y. Sakai, T. Yoshida, Y. Nagai, The paramyxovirus, Sendai virus, V protein encodes a luxury function required for viral pathogenesis, EMBO J. 16 (1997) 578–587.

[18] T. Matano, M. Kano, A. Takeda, H. Nakamura, N. Nomura, Y. Furuta, T. Shioda, Y. Nagai, No significant enhancement of protection by Tat-expressing Sendai viral vector-booster in a macaque AIDS model, AIDS 17 (2003) 1392–1394.

[19] H.O. Li, Y.F. Zhu, M. Asakawa, H. Kuma, T. Hirata, Y. Ueda, Y.S. Lee, M. Fukumura, A. Iida, A. Kato, Y. Nagai, M. Hasegawa, A cytoplasmic RNA vector derived from nontransmissible Sendai virus with efficient gene transfer and expression, J. Virol. 74 (2000) 6564–6569.

[20] M. Yoshizaki, T. Hironaka, H. Iwasaki, H. Ban, Y. Tokusumi, A. Iida, Y. Nagai, M. Hasegawa, M. Inoue, Naked Sendai virus vector lacking all of the envelope-related genes: reduced cytopathogenicity and immunogenicity, J. Gene. Med. 8 (2006) 1151–1159.

[21] K.S. Slobod, J.L. Shenep, J. Lujan-Zilbermann, K. Allison, B. Brown, R.A. Scroggs, A. Portner, C. Coleclough, J.L. Hurwitz, Safety and immunogenicity of intranasal murine parainfluenza virus type 1 (Sendai virus) in healthy human adults, Vaccine 22 (2004) 3182–3186.

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Gag-Specific Cytotoxic T-Lymphocyte-Based Control of Primary Simian Immunodeficiency Virus Replication in a Vaccine Trial[▽]

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Gag-specific cytotoxic T lymphocytes (CTLs) exert strong suppressive pressure on human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) replication. However, it has remained unclear whether they can actually contain primary viral replication. Recent trials of prophylactic vaccines inducing virus-specific T-cell responses have indicated their potential to confer resistance against primary SIV replication in rhesus macaques, while the immunological determinant for this vaccine-based viral control has not been elucidated thus far. Here we present evidence implicating Gag-specific CTLs as responsible for the vaccine-based primary SIV control. Prophylactic vaccination using a Gag-expressing Sendai virus vector resulted in containment of SIVmac239 challenge in all rhesus macaques possessing the major histocompatibility complex (MHC) haplotype *90-120-Ia*. In contrast, *90-120-Ia*-positive vaccinees failed to contain SIVs carrying multiple *gag* CTL escape mutations that had been selected, at the cost of viral fitness, in SIVmac239-infected *90-120-Ia*-positive macaques. These results show that Gag-specific CTL responses do play a crucial role in the control of wild-type SIVmac239 replication in vaccinees. This study implies the possibility of Gag-specific CTL-based primary HIV containment by prophylactic vaccination, although it also suggests that CTL-based AIDS vaccine efficacy may be abrogated in viral transmission between MHC-matched individuals.

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Despite tremendous efforts to develop AIDS vaccines eliciting virus-specific T-cell responses, whether this approach actually does result in controlling human immunodeficiency virus (HIV) replication remains unknown. Recent trials have shown reductions in postchallenge viral loads by prophylactic vaccination eliciting virus-specific T-cell responses in macaque AIDS models (19, 22, 34), but the first advanced human trial of a T-cell-based vaccine was halted because of a lack of efficacy (5). Hence, it is quite important to determine which T-cell responses are responsible for primary HIV control.

Cytotoxic T-lymphocyte (CTL) responses have been indicated to play an important role in the control of HIV and simian immunodeficiency virus (SIV) infections (2, 9, 10, 17, 23, 29). Above all, the potential of Gag-specific CTL responses to contribute to viral control has been suggested by a cohort study indicating an association of HIV control with the breadth of Gag-specific CTL responses (15). In support of this, a recent in vitro study revealed their ability to rapidly respond to SIV infection (28). However, it has remained unclear whether Gag-specific CTL-based viral containment can be achieved by prophylactic vaccination.

We previously developed a prophylactic AIDS vaccine regimen consisting of a DNA prime followed by a boost with a Sendai virus (SeV) vector expressing SIVmac239 Gag (SeV-Gag) (22, 32). Our trial showed potential for efficiently inducing Gag-specific T-cell responses and containment of SIVmac239 challenge in a group of Burmese rhesus macaques sharing the major histocompatibility complex class I (MHC-I) haplotype *90-120-Ia* (22). A follow-up study revealed the reappearance of plasma viremia at >1 year postchallenge in some of these *90-120-Ia*-positive SIV controllers. In these transient controllers, multiple CTL escape mutations were accumulated in the viral *gag* gene, resulting in viremia reappearance and thus suggesting the involvement of $Gag_{206-216}$ (IINEEAADWDL) epitope-specific, $Gag_{241-249}$ (SSVDEQIQW) epitope-specific, and $Gag_{373-380}$ (APVPIPFA) epitope-specific CTLs in sustained viral control (12). Nonetheless, it has remained undetermined whether such Gag-specific CTL responses were responsible for the vaccine-based primary SIV control in *90-120-Ia*-positive vaccinees. In the present study, we challenged the *90-120-Ia*-positive vaccinees with SIVs carrying the *gag* CTL escape mutations to determine the role of Gag-specific CTLs in primary SIVmac239 control.

MATERIALS AND METHODS

Viral competition assay. SIV molecular clone DNAs with *gag* mutations were constructed by site-directed mutagenesis from the wild-type SIVmac239 (14) molecular clone DNA. Virus stocks were obtained by transfection of COS-1 cells with wild-type or mutant SIV molecular clone DNAs, and their titers were

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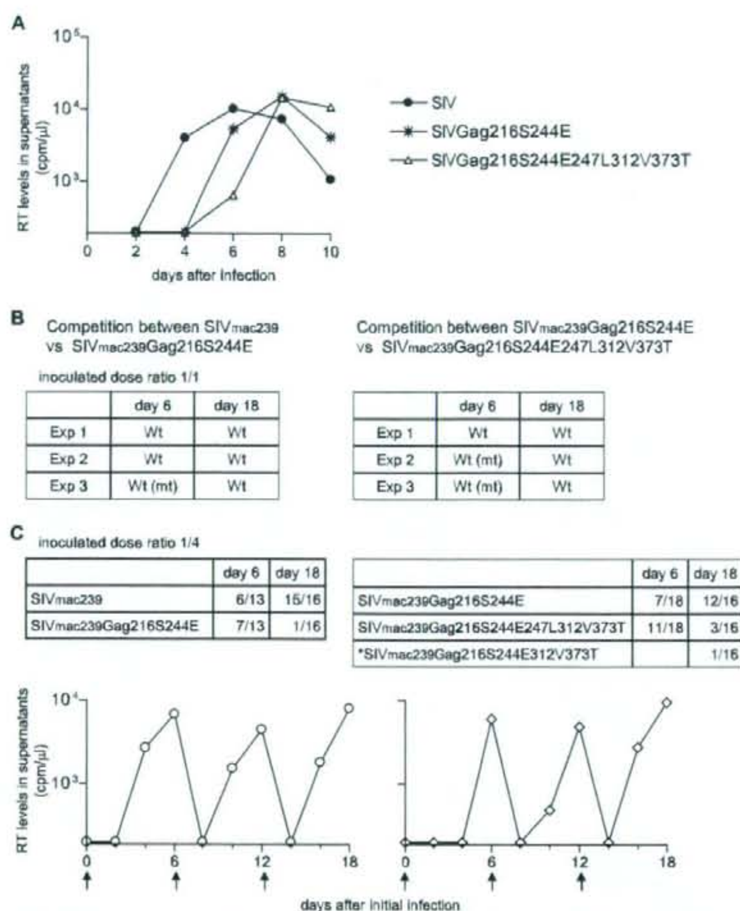

B, left (Competition between SIVmac239 vs SIVmac239Gag216S244E; inoculated dose ratio 1/1):

	day 6	day 18
Exp 1	Wt	Wt
Exp 2	Wt	Wt
Exp 3	Wt (mt)	Wt

B, right (Competition between SIVmac239Gag216S244E vs SIVmac239Gag216S244E247L312V373T):

	day 6	day 18
Exp 1	Wt	Wt
Exp 2	Wt (mt)	Wt
Exp 3	Wt (mt)	Wt

C, left (inoculated dose ratio 1/4):

	day 6	day 18
SIVmac239	6/13	15/16
SIVmac239Gag216S244E	7/13	1/16

C, right:

	day 6	day 18
SIVmac239Gag216S244E	7/18	12/16
SIVmac239Gag216S244E247L312V373T	11/18	3/16
*SIVmac239Gag216S244E312V373T		1/16

FIG. 1. Replication of mutant SIVs in vitro. (A) Wild-type and mutant SIV replication kinetics in HSC-F cells. HSC-F cells were infected with SIVmac239 (closed circles), SIVmac239Gag216S244E (asterisks), or SIVmac239Gag216S244E247L312V373T (open triangles). Virus production was monitored by measuring RT activity in the culture supernatants. Representative results from three sets of experiments are shown. (B) Viral competition assay. HSC-F cells were coinfected with SIVmac239 and SIVmac239Gag216S244E (left) or with SIVmac239Gag216S244E and SIV mac239Gag216S244E247L312V373T (right) at a ratio of 1:1. Viral *gag* fragments were amplified by RT-PCR from viral RNAs from the culture supernatants at days 6 and 18 postinfection and then sequenced. Dominant amino acid sequences at the 216th and 244th aa (left) or the 247th, 312th, and 373rd aa (right) in Gag in three sets of experiments are shown. Wt, only the wild-type sequence was detected; Wt (mt), the wild type was dominant, but the mutant was detectable (the mutant/wild-type ratio was $<1/2$). (C) Viral competition assay. HSC-F cells were coinfected with SIVmac239 and SIVmac239Gag216S244E (left) or with SIVmac239Gag216S244E and SIVmac239Gag216S244E247L312V373T (right) at a ratio of 1:4. The amplified *gag* fragments were subcloned into plasmids and sequenced. Frequencies of the indicated SIV clones (number of indicated clone per total number of clones) are shown. Changes in RT levels in the culture supernatants are shown in the bottom panels. The arrows indicate the time points of coinfection (at day 0) and viral passage for the second (at day 6) and the third (at day 12) cultures.

measured by reverse transcription (RT) assay as described previously (25, 33). For analysis of viral replication, HSC-F cells (herpesvirus saimiri-immortalized macaque T-cell line) (1) were infected with wild-type or mutant SIVs (normalized by RT activity), and virus production was monitored by measuring RT activity in the culture supernatants. For competition, HSC-F cells were coinfected with two SIVs at a ratio of 1:1 or 1:4, and the culture supernatants were harvested every other day and used for RT assays. On day 6, the supernatant was added to fresh HSC-F cells to start the second culture. Similarly, on day 12 after the initial coinfection, the second culture supernatant was added to fresh HSC-F cells to start the third culture. RNAs were extracted from the initial culture supernatant on day 6 and from the third culture supernatant on day 18 postcoinfection. The fragment (nucleotides 1231 to 2958 in SIVmac239 [GenBank accession number M33262]) containing the entire *gag* region was amplified from the RNA by RT-PCR and sequenced. Alternatively, it was subcloned into plasmids to determine dominant sequences.

Animal experiments. Burmese rhesus macaques (*Macaca mulatta*) were maintained in accordance with the guidelines for animal experiments performed at the National Institute of Infectious Diseases (26). Three animals, R01-007, R02-003, and R02-012, that received a prophylactic DNA prime/SeV-Gag boost vaccine and contained SIVmac239 challenge have been reported previously (22). In the present study, macaques R06-015, R06-035, R06-041, R05-004, R05-027, and R07-005 also received the DNA prime/SeV-Gag boost vaccine. The DNA used for the vaccination, CMV-SHIVdEN, was constructed from *env*- and *nef*-deleted simian-human immunodeficiency virus $SHIV_{MD14YE}$ molecular clone

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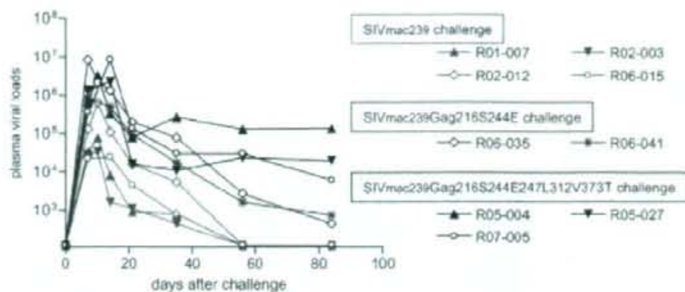

FIG. 2. Plasma viral loads after wild-type or mutant SIV challenge. The *90-120-Ia*-positive vaccinees were challenged with SIVmac239 (red lines), SIVmac239Gag216S244E (blue lines), or SIVmac239Gag216S244E247L312V373T (black lines). Plasma viral loads (SIV *gag* RNA copies/ml plasma) were determined as described before (22). The lower limit of detection is approximately 4×10^2 copies/ml.

DNA (SIVGP1) (31, 32) 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 step, animals received a single boost intranasally with 6×10^9 cell infectious units of F-deleted replication-defective SeV-Gag (21, 32). Approximately 3 months after the boost, animals were challenged intravenously with 1,000 50% tissue culture infective doses of SIVmac239, SIVmac239Gag216S244E, or SIVmac239Gag216S244E247L312V373T. The challenge virus stocks were prepared by virus propagation on rhesus macaque peripheral blood mononuclear cells (PBMCs). Sequence analysis confirmed the absence of *gag* mutations except for the two or five mutations in the challenge viruses.

Immunostaining of CD4$^+$ T-cell memory subsets. PBMCs were subjected to immunofluorescence staining by using fluorescein isothiocyanate-conjugated anti-human CD28, phycoerythrin-conjugated anti-human CD95, peridinin chlorophyll protein-conjugated anti-human CD4, and allophycocyanin-conjugated anti-human CD3 monoclonal antibodies (Becton Dickinson, Tokyo, Japan). The central memory subset of CD4$^+$ T cells was defined by possession of a CD28$^+$ CD95$^+$ phenotype, as described previously (13, 27).

Measurement of virus-specific CD8$^+$ T-cell responses. We measured virus-specific CD8$^+$ T-cell levels by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific stimulation, as described previously (13, 22). In brief, PBMCs were cocultured with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines infected with a vaccinia virus vector expressing SIVmac239 Gag for Gag-specific stimulation or a vesicular stomatitis virus G protein-pseudotyped SIVGP1 for SIV-specific stimulation. The pseudotyped virus was obtained by cotransfection of COS-1 cells with a vesicular stomatitis virus G protein expression plasmid and the SIVGP1 DNA. Alternatively, B-lymphoblastoid cell lines were pulsed with 1 to 10 μM peptides for peptide-specific stimulation (11, 12). The 15-mer $Gag_{367-381}$ peptide was used to detect $Gag_{367-381}$-specific CTLs, including $Gag_{373-380}$-specific CTLs. Intracellular IFN-γ staining was performed using a Cytofix Cytoperm kit (Becton Dickinson). Peridinin chlorophyll protein-conjugated anti-human CD8, allophycocyanin-conjugated anti-human CD3, and phycoerythrin-conjugated anti-human IFN-γ antibodies (Becton Dickinson) were used. Specific T-cell levels were calculated by subtracting nonspecific IFN-γ$^+$ T-cell frequencies from those after Gag-specific, SIV-specific, or peptide-specific stimulation. Specific T-cell levels of <100 cells per million PBMCs were considered negative.

Statistical analysis. Statistical analysis was performed with Prism software, version 4.03, with significance set at P values of <0.05 (GraphPad Software, Inc., San Diego, CA). Central memory CD4$^+$ T-cell counts before challenge were not significantly different between the wild-type SIV-challenged ($n = 4$) and the mutant SIV-challenged ($n = 5$) macaques ($P = 0.70$ by unpaired two-tailed t test with Welch's correction and $P = 0.73$ by nonparametric Mann-Whitney U test). Ratios of the central memory CD4$^+$ T-cell counts from a few months postchallenge to those prechallenge were log transformed and compared between the two groups by an unpaired two-tailed t test and the Mann-Whitney U test. Gag-specific CD8$^+$ T-cell frequencies postvaccination (prechallenge) or postchallenge were also log transformed and compared between the two groups in the same statistical manner.

RESULTS

Comparison of viral fitness in wild-type and mutant SIVs. We used two mutant SIVs for challenge of the *90-120-Ia*-positive vaccinees. The first, designated SIVmac239Gag216S244E, carries two *gag* mutations, GagL216S and GagD244E, leading to a leucine (L)-to-serine (S) substitution at the 216th amino acid (aa) and an aspartic acid (D)-to-glutamic acid (E) substitution at the 244th aa in Gag. The second, designated SIVmac239Gag216S244E247L312V373T, carries five *gag* mutations, GagL216S, GagD244E, GagI247L (isoleucine [I] to L at the 247th aa), GagA312V (alanine [A] to valine [V] at the 312th aa), and GagA373T (A to threonine [T] at the 373rd aa). In our previous study (12), the former became dominant in the early phase (at approximately 4 months postchallenge) during the period of viral control, and the latter was dominant at viremia reappearance in a transient controller. GagL216S, GagD244E and GagI247L, and GagA373T mutations result in viral escape from recognition by $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, and $Gag_{373-380}$-specific CTLs, respectively, while it remains unclear whether GagA312V was selected for by CTLs.

We first compared viral fitness in wild-type and mutant SIVs. In HSC-F cells (a macaque T-cell line), not only the wild type but also the mutant SIVs were able to replicate, but SIVmac239Gag216S244E replication was less efficient than that of wild-type SIVmac239, and SIVmac239Gag216S244E247L312V373T replication was even less efficient (Fig. 1A). In competitions between two SIVs, HSC-F cells were coinfected with both viruses, and viral genome sequences in the culture supernatants were assessed to establish which SIV became predominant. In culture supernatants of HSC-F cells after coinfection with SIVmac239 and SIVmac239Gag216S244E inoculated at a ratio of 1:1, the wild type rapidly became dominant (at day 6) (Fig. 1B). Coinfection at a ratio of 1:4 resulted in equivalence at day 6, but the wild type again dominated by day 18 (Fig. 1C). These results indicate a lower replicative ability of SIVmac239Gag216S244E than of wild-type SIVmac239. In addition, competition between SIVmac239Gag216S244E and SIVmac239Gag216S244E247L312V373T showed the lower replicative ability of the latter (Fig. 1B and C).

Challenge of *90-120-Ia*-positive vaccinees with wild-type or mutant SIVs. Next, we challenged *90-120-Ia*-positive macaques

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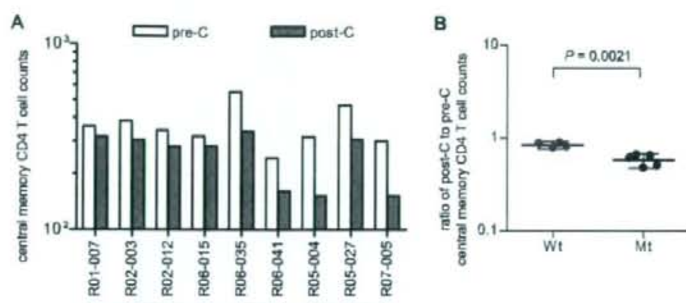

FIG. 3. Changes in central memory CD4$^+$ T-cell counts after wild-type or mutant SIV challenge. (A) Peripheral central memory CD4$^+$ (CD4$^+$ CD95$^+$ CD28$^+$) T-cell counts (/μl) prechallenge (pre-C) and a few months postchallenge (post-C). (B) Statistical comparison of central memory CD4$^+$ T-cell loss between the wild-type SIV-challenged (Wt) and the mutant SIV-challenged (Mt) macaques. The ratios of central memory CD4$^+$ T-cell counts postchallenge to those prechallenge are plotted. The longer bars indicate geometric mean values, and the regions between the shorter bars indicate the 95% confidence intervals. The ratios in the mutant group (n = 5) were significantly lower than those in the wild-type group (n = 4) (P = 0.0021 by unpaired t test and P = 0.0159 by Mann-Whitney U test).

with the mutant SIVs after DNA prime/SeV-Gag vaccination. Remarkably, all three vaccinees (R05-004, R05-027, and R07-005) challenged with SIVmac239Gag216S244E247L312V373T failed to control viral replication and showed high set point plasma viral loads, while all four vaccinees (R01-007, R02-003, R02-012, and R06-015) challenged with wild-type SIVmac239 contained viral replication, with undetectable set point plasma viral loads (Fig. 2). Even the two vaccinees (R06-035 and R06-041) challenged with SIVmac239Gag216S244E failed to contain viral replication, although with lower plasma viral loads, at approximately 10^3 RNA copies/ml at 3 months postchallenge. Central memory CD4$^+$ T-cell counts before challenge were not significantly different between the wild-type SIV-challenged (n = 4) and mutant SIV-challenged (n = 5) macaques, but ratios of the counts at a few months postchallenge to prechallenge for the latter group were significantly lower than those for the former (P = 0.0021 by unpaired t test and P = 0.0159 by Mann-Whitney U test) (Fig. 3). Thus, *90-120-Ia*-positive vaccinees can contain wild-type SIVmac239 but not SIVmac239Gag216S244E or SIVmac239Gag216S244E247L312V373T challenge.

Viral *gag* sequence analysis confirmed the rapid selection for the GagL216S mutation in all wild-type SIVmac239-challenged macaques, as described previously (22). All of the *gag* mutations in the challenge mutant viruses were maintained during the observation period (Table 1). SIVmac239Gag216S244E247L312V373T-challenged macaques showed no additional dominant *gag* mutations, whereas animals challenged with SIVmac239Gag216S244E rapidly selected viruses with a GagV145A (V to A at the 145th aa) mutation. Recovery of viral fitness by this mutation was not observed, and whether it was selected for by CTLs was unclear in our previous study (12).

Gag-specific CTL responses were induced after SeV-Gag boost in all vaccinees, and there was no significant difference in the levels between the wild-type and mutant challenges (P = 0.1198 by unpaired t test and P = 0.1111 by Mann-Whitney U test). However, secondary Gag-specific CTL responses were

TABLE 1. Dominant sequences in SIV Gag in macaques after challenge

Macaque	Time (wk) of plasma sample	Amino acid change in Gag at position[a]:								
		140	145	206	216	244	247	312	341	373
R01-007	5				L216S					
R02-003	5				L216S					
R02-012	5				L216S					
R06-015	5			(I206M)	L216S					
R06-035	5				L216S*	D244E*				
	12		V145A		L216S*	D244E*			(N341Y)	
R06-041	5		(V145A)		L216S*	D244E*				
	12		V145A		L216S*	D244E*				
R05-004	5				L216S*	D244E*	I247L*	A312V*		A373T*
	12	(I140V)			L216S*	D244E*	I247L*	A312V*		A373T*
R05-027	5				L216S*	D244E*	I247L*	A312V*		A373T*
	12				L216S*	D244E*	I247L*	A312V*		A373T*
R07-005	5				L216S*	D244E*	I247L*	A312V*		A373T*
	12				L216S*	D244E*	I247L*	A312V*		A373T*

[a] A fragment containing the entire *gag* region was amplified from plasma RNA by nested RT-PCR and then sequenced. We were unable to amplify the fragment from plasmas obtained at week 12 from the wild-type SIVmac239-challenged macaques with undetectable viremia. Dominant *gag* mutations resulting in amino acid changes are shown. Asterisks indicate the mutations included in the challenge inoculums. Parentheses indicate that both the wild-type and mutant sequences were detected equivalently at that position.

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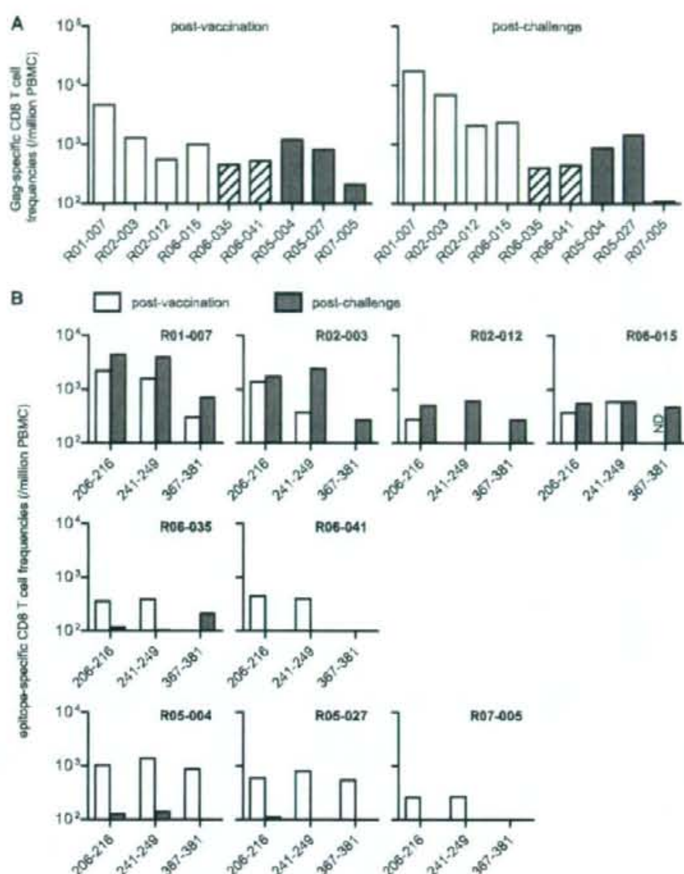

FIG. 4. Gag-specific CD8+ T-cell responses before and after wild-type or mutant SIV challenge. Macaques R01-007, R02-003, R02-012, and R06-015 were challenged with SIVmac239; macaques R06-035 and R06-041 were challenged with SIVmac239Gag216S244E; and macaques R05-004, R05-027, and R07-005 were challenged with SIVmac239Gag216S244E247L312V373T. (A) Gag-specific CD8+ T-cell frequencies at 2 weeks postboost (postvaccination) (left) and 2 weeks postchallenge (right). (B) $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, and $Gag_{367-381}$-specific CD8+ T-cell frequencies at 2 weeks (all except for R02-012) or 4 weeks (in R02-012) postboost (postvaccination) and 5 weeks (in R01-007, R02-003, R02-012, R06-035, R06-041, and R05-004) or 6 weeks (in R06-015, R05-027, and R07-005) postchallenge. ND, not determined.

less efficient after challenge with mutant SIV than after challenge with wild-type SIV ($P = 0.0095$ by unpaired t test and $P = 0.0159$ by Mann-Whitney U test) (Fig. 4A).

SeV-Gag boost induced efficient $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses in all vaccinees and $Gag_{367-381}$-specific CTL responses in some of them (Fig. 4B). Challenge with wild-type SIVmac239 resulted in efficient secondary responses of these three epitope-specific CTLs, whereas SIVmac 239Gag216S244E247L312V373T challenge evoked none of them (Fig. 4B). SIVmac239Gag216S244E challenge did not result in secondary responses of $Gag_{206-216}$-specific or $Gag_{241-249}$-specific CTLs but did induce $Gag_{367-381}$-specific CTL responses in one case (Fig. 4B). These results indicate that SIVmac239 Gag216S244E evades recognition by $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTLs and that SIVmac239Gag216S244E247L312V373T evades recognition by $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, and $Gag_{367-381}$-specific CTLs.

We next examined Gag-specific and SIV-specific CTL responses after mutant SIV challenge (Fig. 5A). We used an *env*- and *nef*-deleted SHIV molecular clone DNA, SIVGP1, that has the genes encoding SIVmac239 Gag, Pol, Vif, Vpx, and a part of Vpr and measured the frequencies of CTLs responding to SIVGP1-transduced cells (referred to as SIV-specific CTLs) as described previously (13, 32). SIV-specific CTL frequencies at week 12 were much higher than those at week 2 for all five macaques challenged with mutant SIVs. In contrast, Gag-specific CTL frequencies at week 12 were lower than those at week 2 for four of five animals; the remaining macaque, R06-035, mounted $Gag_{367-381}$-specific CTL responses. Importantly, in all animals challenged with mutant SIVs, SIV-specific CTL

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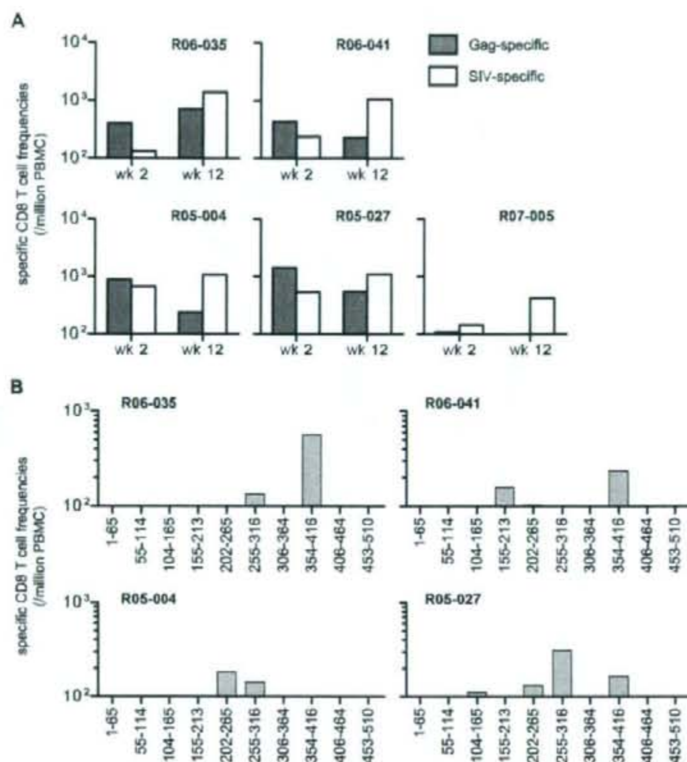

FIG. 5. SIV-specific CD8+ T-cell responses after mutant SIV challenge. (A) Gag-specific (closed boxes) and SIV-specific (open boxes) CD8+ T-cell frequencies at 2 weeks or 12 weeks postchallenge. (B) Frequencies of CD8+ T cells specific for pools of SIV Gag peptides. A panel of 117 overlapping peptides (15 to 17 aa in length and overlapping by 10 to 12 aa) spanning the entire SIV Gag amino acid sequence were divided into the following 10 pools (each consisting of 11 or 12 peptides): pool 1, 1st to 65th aa in SIV Gag; pool 2, 55th to 114th aa; pool 3, 104th to 165th aa; pool 4, 155th to 213th aa; pool 5, 202nd to 265th aa; pool 6, 255th to 316th aa; pool 7, 306th to 364th aa; pool 8, 354th to 416th aa; pool 9, 406th to 464th aa; and pool 10, 453rd to 510th aa. The pools were used for stimulation to detect peptide pool-specific CD8+ T cells.

frequencies were at marginal levels or lower than Gag-specific CTL frequencies at week 2, but the former became higher than the latter at week 12. These results indicate an induction of CTL responses specific for SIV antigens other than Gag in all five macaques after mutant SIV challenge.

At week 12 after mutant SIV challenge, Gag-specific CTL responses were undetectable in macaque R07-005 but were still detected in the other four macaques. We then analyzed Gag-specific CTL responses in these four macaques by using a panel of overlapping peptides spanning the entire SIV Gag amino acid sequence (Fig. 5B). In both SIVmac239Gag216S244E-challenged animals, R06-035 and R06-041, exhibiting detectable $Gag_{367-381}$-specific CTL responses (data not shown). CTL responses specific for the peptide mixture corresponding to the 354th to 416th aa in SIV Gag were detected at week 12. In addition, we found $Gag_{255-316}$-specific CTL responses in macaque R06-035 and $Gag_{155-213}$-specific CTL responses in macaque R06-041. SIVmac239Gag216S244E247L312V373T-challenged macaques R05-004 and R05-027 showed responses specific for several Gag peptide mixtures, including $Gag_{202-265}$-specific and $Gag_{255-316}$-specific CTL responses. These results indicate an induction of CTL responses specific for Gag epitopes other than the $Gag_{206-216}$, $Gag_{241-249}$, and $Gag_{373-380}$ epitopes after mutant SIV challenge.

DISCUSSION

In the present study, SIVs carrying multiple *gag* CTL escape mutations showed lower replicative abilities than that of the wild type; nonetheless, the *90-120-Ia*-positive vaccinees were able to contain only the latter. This demonstrates that Gag-specific CTL responses did play a central role in the vaccine-based primary containment of wild-type SIVmac239 replication in *90-120-Ia*-positive macaques.

Elicitation of virus-specific T-cell responses by prophylactic vaccination is believed to be a promising strategy for HIV control (3, 24); whether this approach can actually result in HIV control remains unknown. Recent studies have indicated the possibility of reductions in set point viral loads after SIV challenge by prophylactic vaccination inducing T-cell responses in rhesus macaques (19, 22, 34), yet the immune component crucial for the vaccine-based viral control has not been

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determined. No clear evidence for a contribution of vaccine-induced CTLs to this viral control has been forthcoming to date, although virus-specific CTL responses have been implicated in exerting strong suppressive pressure on HIV/SIV infection (9, 22). Indeed, viral replication persists even in the presence of CTL responses in the natural course of infection; it has thus remained unclear whether HIV/SIV replication can be controlled by vaccine-induced CTLs. The evidence from the present study now strongly implicates Gag-specific CTL responses as responsible for vaccine-based primary SIV control. This offers the possibility of Gag-specific CTL-based HIV containment by prophylactic vaccination and provides insight into the development of CTL-based AIDS vaccines.

The containment of SIVmac239 but failure to contain SIV mac239Gag216S244E in the vaccinees documents a crucial role for $Gag_{206-216}$-specific and/or $Gag_{241-249}$-specific CTL responses in vaccine-based SIVmac239 containment. Furthermore, challenge with SIVmac239Gag216S244E247L312V373T, possessing diminished viral fitness compared to SIVmac239Gag216S244E, tended to result in higher viral loads, indicating the involvement of $Gag_{373-380}$-specific CTL responses in viral control, while more complete viral evasion of $Gag_{241-249}$-specific CTL recognition by addition of the GagI247L mutation may also contribute to the difference between SIVmac239 Gag216S244E and SIVmac239Gag216S244E247L312V373T challenge. Taken together, we conclude that these two or three epitope-specific CTL responses are crucial for primary SIVmac239 control in *90-120-Ia*-positive vaccinees. Conversely, this study implies that viral evasion of recognition by two dominant epitope-specific CTLs can result in failure of primary viral containment but may not be sufficient for abrogation of vaccine efficacy. Thus, analysis of CTL-based vaccine efficacy against SIVs carrying single or multiple CTL escape mutations could contribute to an evaluation of its potential for controlling the replication of highly diversified HIVs.

Our results suggest that SIV- but non-Gag-specific CTLs became predominant after mutant SIV challenge. Additionally, CTLs recognizing Gag regions other than the $Gag_{206-216}$, $Gag_{241-249}$, and $Gag_{373-380}$ epitopes were detected in most cases. These CTL responses may exert suppressive pressure on viral replication but are considered insufficient for controlling replication of the mutant SIVs with lower viral fitness.

Finally, this study also provides evidence indicating a possible abrogation of CTL-based AIDS vaccine efficacy in viral transmission between MHC-I-matched individuals. Indeed, even the mutant SIVs carrying multiple CTL escape mutations were able to replicate persistently in vivo, despite their diminished replicative ability. Transmission of these viruses can result in persistent viral infection and AIDS progression (30). CTL escape mutations resulting in a loss of viral fitness may revert to the wild-type sequence after transmission into MHC-I-mismatched hosts (4, 8, 9, 16, 18, 20), but such reversion does not occur rapidly; alternatively, some may be retained with additional compensatory mutations (6, 7, 30). Thus, there may be a risk of transmission and accumulation of HIV CTL escape variants even among MHC-I-mismatched individuals, resulting in abrogation of CTL-based AIDS vaccine efficacy in a population.

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REFERENCES

1. **Akari, H., K. Mori, K. Terao, I. Otani, M. Fukasawa, R. Mukai, and Y. Yoshikawa.** 1996. In vitro immortalization of Old World monkey T lymphocytes with herpesvirus saimiri: its susceptibility to infection with simian immunodeficiency viruses. Virology **218:**382–388.
2. **Borrow, P., H. Lewicki, B. H. Hahn, G. M. Shaw, and M. B. Oldstone.** 1994. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. J. Virol. **68:**6103–6110.
3. **Brander, C., and B. D. Walker.** 1999. T lymphocyte responses in HIV-1 infection: implication for vaccine development. Curr. Opin. Immunol. **11:**451–459.
4. **Brander, C., and B. D. Walker.** 2003. Gradual adaptation of HIV to human host populations: good or bad news? Nat. Med. **9:**1359–1362.
5. **Cohen, J.** 2007. Did Merck's failed HIV vaccine cause harm? Science **318:**1048–1049.
6. **Crawford, H., J. G. Prado, A. Leslie, S. Hué, I. Honeyborne, S. Reddy, M. van der Stok, Z. Mncube, C. Brander, C. Rousseau, J. I. Mullins, R. Kaslow, P. Goepfert, S. Allen, E. Hunter, J. Mulenga, P. Kiepiela, B. D. Walker, and P. J. R. Goulder.** 2007. Compensatory mutation partially restores fitness and delays reversion of escape mutation within the immunodominant HLA-B*5703-restricted Gag epitope in chronic human immunodeficiency virus type 1 infection. J. Virol. **81:**8346–8351.
7. **Friedrich, T. C., C. A. Frye, L. J. Yant, D. H. O'Connor, N. A. Kriewaldt, M. Benson, L. Vojnov, E. J. Dodds, C. Cullen, R. Rudersdorf, A. L. Hughes, N. Wilson, and D. I. Watkins.** 2004. Extra-epitopic compensatory substitutions partially restore fitness to simian immunodeficiency virus variants that escape from an immunodominant cytotoxic T-lymphocyte response. J. Virol. **78:**2581–2585.
8. **Friedrich, T. C., E. J. Dodds, L. J. Yant, L. Vojnov, R. Rudersdorf, C. Cullen, D. T. Evans, R. C. Desrosiers, B. R. Mothe, J. Sidney, A. Sette, K. Kunstman, S. Wolinsky, M. Piatak, J. Lifson, A. L. Hughes, N. Wilson, D. H. O'Connor, and D. I. Watkins.** 2004. Reversion of CTL escape-variant immunodeficiency viruses in vivo. Nat. Med. **10:**275–281.
9. **Goulder, P. J., and D. I. Watkins.** 2004. HIV and SIV CTL escape: implications for vaccine design. Nat. Rev. Immunol. **4:**630–640.
10. **Jin, X., D. E. Bauer, S. E. Tuttleton, S. Lewin, A. Gettie, J. Blanchard, C. E. Irwin, J. T. Safrit, J. Mittler, L. Weinberger, L. G. Kostrikis, L. Zhang, A. S. Perelson, and D. D. Ho.** 1999. Dramatic rise in plasma viremia after CD8+ T cell depletion in simian immunodeficiency virus-infected macaques. J. Exp. Med. **189:**991–998.
11. **Kato, M., H. Igarashi, A. Takeda, Y. Sasaki, H. Nakamura, M. Kano, T. Sata, A. Iida, M. Hasegawa, S. Horie, E. Higashihara, Y. Nagai, and T. Matano.** 2005. Induction of Gag-specific T-cell responses by therapeutic immunization with a Gag-expressing Sendai virus vector in macaques chronically infected with simian-human immunodeficiency virus. Vaccine **23:**3166–3173.
12. **Kawada, M., H. Igarashi, A. Takeda, T. Tsukamoto, H. Yamamoto, S. Dohki, M. Takiguchi, and T. Matano.** 2006. Involvement of multiple epitope-specific cytotoxic T-lymphocyte responses in vaccine-based control of simian immunodeficiency virus replication in rhesus macaques. J. Virol. **80:**1949–1958.
13. **Kawada, M., T. Tsukamoto, H. Yamamoto, A. Takeda, H. Igarashi, D. I. Watkins, and T. Matano.** 2007. Long-term control of simian immunodeficiency virus replication with central memory CD4+ T-cell preservation after nonsterile protection by a cytotoxic T-lymphocyte-based vaccine. J. Virol. **81:**5202–5211.
14. **Kestler, H. W., III, D. J. Ringler, K. Mori, D. L. Panicali, P. K. Sehgal, M. D. Daniel, and R. C. Desrosiers.** 1991. Importance of the nef gene for maintenance of high virus loads and for development of AIDS. Cell **65:**651–662.
15. **Kiepiela, P., K. Ngumbela, C. Thobakgale, D. Ramduth, I. Honeyborne, E. Moodley, S. Reddy, C. de Pierres, Z. Mncube, N. Mkhwanazi, K. Bishop, M. van der Stok, K. Nair, N. Khan, H. Crawford, R. Payne, A. Leslie, J. Prado, A. Prendergast, J. Frater, N. McCarthy, C. Brander, G. H. Learn, D. Nickle, C. Rousseau, H. Coovadia, J. I. Mullins, D. Heckerman, B. D. Walker, and P. Goulder.** 2007. CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. Nat. Med. **13:**46–53.

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16. **Kobayashi, M., H. Igarashi, A. Takeda, M. Kato, and T. Matano.** 2005. Reversion in vivo after inoculation of a molecular proviral DNA clone of simian immunodeficiency virus with a cytotoxic-T-lymphocyte escape mutation. J. Virol. **79:**11529–11532.
17. **Koup, R. A., J. T. Safrit, Y. Cao, C. A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, and D. D. Ho.** 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J. Virol. **68:**4650–4655.
18. **Leslie, A. J., K. J. Pfafferott, P. Chetty, R. Draenert, M. M. Addo, M. Feeney, Y. Tang, E. C. Holmes, T. Allen, J. G. Prado, M. Altfeld, C. Brander, C. Dixon, D. Ramduth, P. Jeena, S. A. Thomas, A. St. John, T. A. Roach, B. Kupfer, G. Luzzi, A. Edwards, G. Taylor, H. Lyall, G. Tudor-Williams, V. Novelli, J. Martinez-Picado, P. Kiepiela, B. D. Walker, and P. J. Goulder.** 2004. HIV evolution: CTL escape mutation and reversion after transmission. Nat. Med. **10:**282–289.
19. **Letvin, N. L., J. R. Mascola, Y. Sun, D. A. Gorgone, A. P. Buzby, L. Xu, Z. Y. Yang, B. Chakrabarti, S. S. Rao, J. E. Schmitz, D. C. Montefiori, B. R. Barker, F. L. Bookstein, and G. J. Nabel.** 2006. Preserved CD4+ central memory T cells and survival in vaccinated SIV-challenged monkeys. Science **312:**1530–1533.
20. **Li, B., A. D. Gladden, M. Altfeld, J. M. Kaldor, D. A. Cooper, A. D. Kelleher, and T. M. Allen.** 2007. Rapid reversion of sequence polymorphisms dominates early human immunodeficiency virus type 1 evolution. J. Virol. **81:**193–201.
21. **Li, H. O., Y. F. Zhu, M. Asakawa, H. Kuma, T. Hirata, Y. Ueda, Y. S. Lee, M. Fukumura, A. Iida, A. Kato, Y. Nagai, and M. Hasegawa.** 2000. A cytoplasmic RNA vector derived from nontransmissible Sendai virus with efficient gene transfer and expression. J. Virol. **74:**6564–6569.
22. **Matano, T., M. Kobayashi, H. Igarashi, A. Takeda, H. Nakamura, M. Kano, C. Sugimoto, K. Mori, A. Iida, T. Hirata, M. Hasegawa, T. Yuasa, M. Miyazawa, Y. Takahashi, M. Yasunami, A. Kimura, D. H. O'Connor, D. I. Watkins, and Y. Nagai.** 2004. Cytotoxic T lymphocyte-based control of simian immunodeficiency virus replication in a preclinical AIDS vaccine trial. J. Exp. Med. **199:**1709–1718.
23. **Matano, T., R. Shibata, C. Siemon, M. Connors, H. C. Lane, and M. A. Martin.** 1998. Administration of an anti-CD8 monoclonal antibody interferes with the clearance of chimeric simian/human immunodeficiency virus during primary infections of rhesus macaques. J. Virol. **72:**164–169.
24. **McMichael, A. J., and T. Hanke.** 2003. HIV vaccines 1983–2003. Nat. Med. **9:**874–880.
25. **Miyagi, E., S. Opi, H. Takeuchi, M. Khan, R. Goila-Gaur, S. Kao, and K. Strebel.** 2007. Enzymatically active APOBEC3G is required for efficient inhibition of human immunodeficiency virus type 1. J. Virol. **81:**13346–13353.
26. **National Institute of Infectious Diseases.** 2007. Guides for animal experiments performed at National Institute of Infectious Diseases. National Institute of Infectious Diseases, Tokyo, Japan. (In Japanese.)
27. **Pitcher, C. J., S. I. Hagen, J. M. Walker, R. Lum, B. L. Mitchell, V. C. Maino, M. K. Axthelm, and L. J. Picker.** 2004. Development and homeostasis of T cell memory in rhesus macaques. J. Immunol. **168:**29–43.
28. **Sacha, J. B., C. Chung, E. G. Rakasz, S. P. Spencer, A. K. Jonas, A. T. Bean, W. Lee, B. J. Burwitz, J. J. Stephany, J. T. Loffredo, D. B. Allison, S. Adnan, A. Hoji, N. A. Wilson, T. C. Friedrich, J. D. Lifson, O. O. Yang, and D. I. Watkins.** 2007. Gag-specific CD8+ T lymphocytes recognize infected cells before AIDS-virus integration and viral protein expression. J. Immunol. **178:**2746–2754.
29. **Schmitz, J. E., M. J. Kuroda, S. Santra, V. G. Sasseville, M. A. Simon, M. A. Lifton, P. Racz, K. Tenner-Racz, M. Dalesandro, B. J. Scallon, J. Ghrayeb, M. A. Forman, D. C. Montefiori, E. P. Rieber, N. L. Letvin, and K. A. Reimann.** 1999. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. Science **283:**857–860.
30. **Seki, S., M. Kawada, A. Takeda, H. Igarashi, T. Sata, and T. Matano.** 2008. Transmission of simian immunodeficiency virus carrying multiple cytotoxic-T-lymphocyte escape mutations with diminished replicative ability can result in AIDS progression in rhesus macaques. J. Virol. **82:**5093–5098.
31. **Shibata, R., F. Maldarelli, C. Siemon, T. Matano, M. Parta, G. Miller, T. Fredrickson, and M. A. Martin.** 1997. Infection and pathogenicity of chimeric simian-human immunodeficiency viruses in macaques: determinants of high virus loads and CD4 cell killing. J. Infect. Dis. **176:**362–373.
32. **Takeda, A., H. Igarashi, H. Nakamura, M. Kano, A. Iida, T. Hirata, M. Hasegawa, Y. Nagai, and T. Matano.** 2003. Protective efficacy of an AIDS vaccine, a single DNA-prime followed by a single booster with a recombinant replication-defective Sendai virus vector, in a macaque AIDS model. J. Virol. **77:**9710–9715.
33. **Willey, R. L., D. H. Smith, L. A. Lasky, T. S. Theodore, P. L. Earl, B. Moss, D. J. Capon, and M. A. Martin.** 1988. In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity. J. Virol. **62:**139–147.
34. **Wilson, N. A., J. Reed, G. S. Napoe, S. Piaskowski, A. Szymanski, J. Furlott, E. J. Gonzalez, L. J. Yant, N. J. Maness, G. E. May, T. Soma, M. R. Reynolds, E. Rakasz, R. Rudersdorf, A. B. McDermott, D. H. O'Connor, T. C. Friedrich, D. B. Allison, A. Patki, L. J. Picker, D. R. Burton, J. Lin, L. Huang, D. Patel, G. Heindecker, J. Fan, M. Citron, M. Horton, F. Wang, X. Liang, J. W. Shiver, D. R. Casimiro, and D. I. Watkins.** 2006. Vaccine-induced cellular immune responses reduce plasma viral concentrations after repeated low-dose challenge with pathogenic simian immunodeficiency virus SIVmac239. J. Virol. **80:**5875–5885.

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