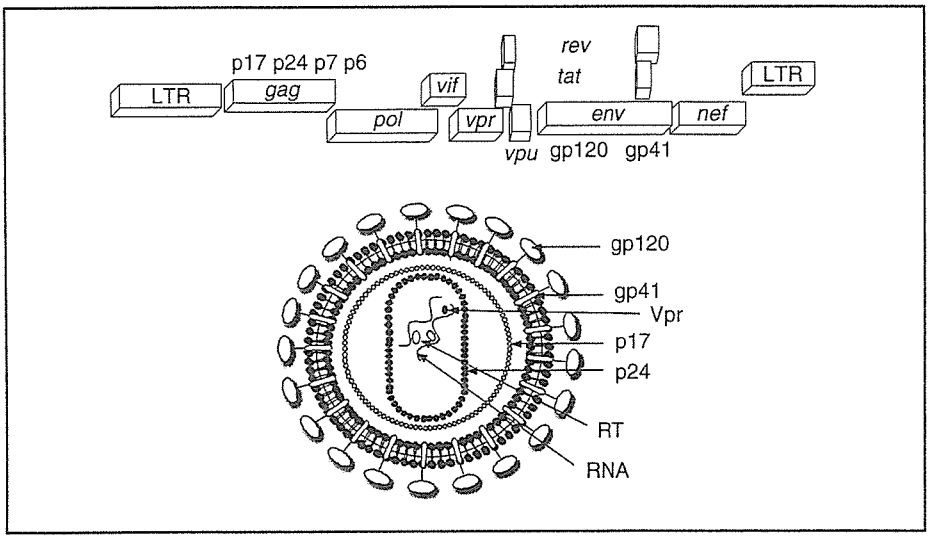


**Fig. 1 The HIV-1 provirus and the virion structure**

The genome consists with the LTRs (long terminal repeat), structural genes (*gag*, *pol*, and *env*), regulatory genes (*tat* and *rev*), and accessories genes (*nef*, *vif*, *vpr*, and *vpu*). The virion consists with Gag, Pol, Env, Vpr, and an RNA dimmer.

occurring due to AIDS has decreased, particularly in advanced countries. Although the incidence of HIV encephalopathy has markedly decreased due to this therapy,[4] in 2004, it was estimated that approximately 3 million patients worldwide continued to die of AIDS. Further, a less severe form of HIV encephalopathy that comprises a milder cognitive and motor disorder (MCMD) is now a potentially serious problem.[5] This syndrome is characterized by a much less pronounced state of memory loss and a decrease in computational and other higher cortical functions. The clinical presence of MCMD has been thought to be associated with the extent of pathological changes observed in the CNS due to HIV invasion. A potential explanation for the development of MCMD is that low level viral replication, as shown even in cases of highly HAART regimens, leads to the gradual progression of neurodegenerative damage.

The CNS of young children appears to be more vulnerable to the effect of HIV than that of adults. This is probably because in young children, the CNS is still in the developmental stage and contains many undifferentiated cells. The progression of pediatric AIDS is rapid and children do not respond to HAART. In addition, clinical analysis reveals that congenitally HIV-infected children frequently develop progressive encephalopathy, which is complicating microcephaly, spastic paraparesis, and delayed developmental milestones. After the introduction of HAART in many areas, the maternal-fetal transmission of HIV has been reduced successfully, thereby reducing the prevalence of progressive encephalopathy.

Currently, more than 6,500 people in Japan have been confirmed to be infected with HIV, and the number of HIV-infected persons is gradually increasing at a rate of 780 patients per year. Although the number of HIV encephalopathy patients clearly decreased after the introduction of the HAART, new problems such as the emergence of HAART-resistant viruses and the side effects of HAART are becoming apparent. Since subclinical MCMD patients appear to be increasing in many countries, HIV encephalopathy may also become a serious disease in Japan.

## HIV and HIV Encephalopathy

HIV is virologically classified into HIV-1 and HIV-2. HIV-1 was initially isolated from a French patient in 1983, and subsequently, it has been identified as the causative agent of AIDS in many other countries. Currently, the HIV-1 epidemic has spread worldwide. It has been demonstrated that HIV-1 originated from chimpanzees:

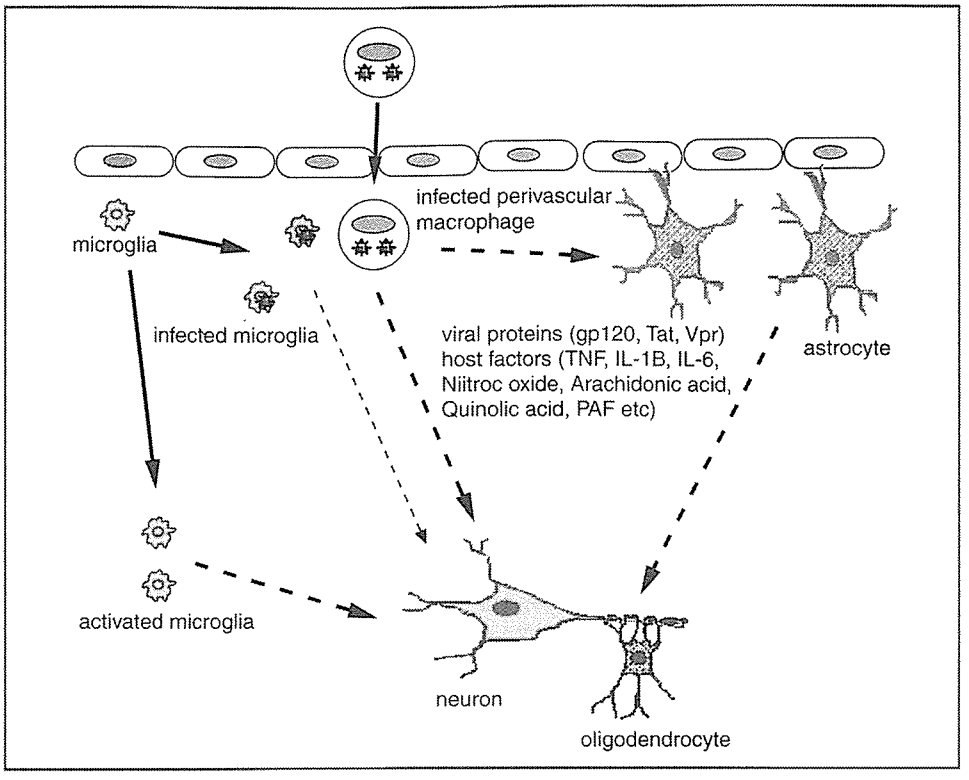


**Fig. 2 Pathogenesis of HIV encephalopathy**

Perivascular macrophages and microglia are responsible for producing HIV. They release viral proteins such as gp120, Tat, and Vpr and other host factors. Simultaneously, microglia and astrocytes are activated by these factors. Neuroprotective and neurodestructive factors coexist in this pathogenesis.

however, infected chimpanzees are resistant to the development of this disease. In 1986, HIV-2 was independently isolated from some patients in West Africa. Interestingly, in the case of HIV-2, it has been demonstrated that it originated in a small monkey species, such as the mangabee, and its potential for causing pathogenesis or acting as a pathogen in humans was clearly low. HIV-1 belongs to the retrovirus family, and its virion structure comprises 100-nm ball-like particles. Two viral RNAs, viral structural proteins, core protein p24, matrix protein p17, nucleocapsid p7, and the accessory protein Vpr are packed into its capsid, and the capsid is enveloped by two viral-encoded glycoproteins, namely, gp120 and gp41, and a plasma membrane-derived lipid (Fig. 1). When the HIV particles attach to the target human cells, gp120 on the viral surface specifically binds to the CD4 molecule on the plasma membrane of the target cells and subsequently to CXCR4 or CCR5, which are the physiological receptor molecules for different chemokines. In vivo, HIV replicates in the $CD4^+$ T cells and macrophages because both these cells express CD4 and CXCR4 or CCR5. Although these receptor positive cells get distributed in many lymphoid tissues such as the peripheral blood and lymph nodes, they rarely reside in the healthy brain. An examination of the autopsy samples of HIV encephalopathy patients revealed that HIV was predominantly found in the macrophages and microglia but not in the $CD4^+$ T cells located near the vessels. It is thought that the $CD4^+$ T cells are depleted in the peripheral blood at the time of development of HIV encephalopathy, and that they invade the brain very little. The typical pathological features of parenchymal infections include the activation of macrophages and astrocytes, cortical and central atrophy, diffuse myelin pallor, multinucleated giant cells, and microglial nodules. Furthermore, the macrophage-tropic virus, which uses CCR5

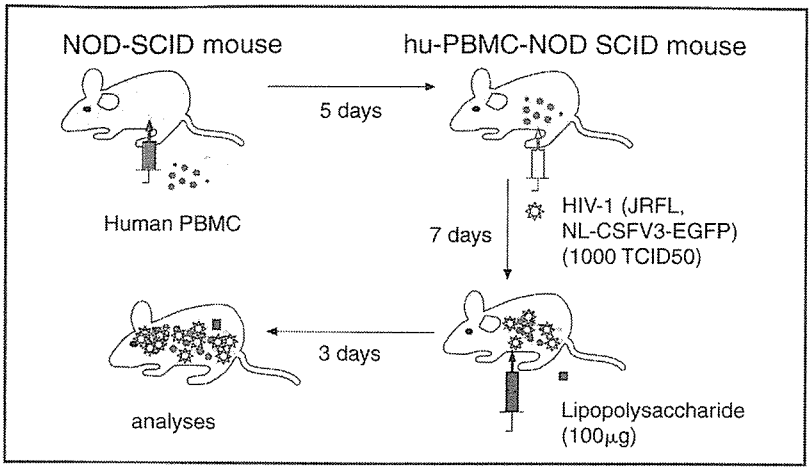


**Fig. 3 A murine model using the hu-PBMC-NOD SCID mouse for HIV encephalopathy**
Human peripheral blood monocytes were intraperitoneally transplanted into the immunodeficient NOD-SCID mouse. Macrophage-tropic HIV-1 and lipopolysaccharide were then intraperitoneally injected into the mouse.

as a coreceptor, is frequently isolated from the HIV-infected brain.[6] However, the T-tropic virus, which uses CXCR4, could not be detected. Interestingly, HIV does not replicate in neurons and oligodendrocytes, which are found to be severely damaged in the infected brains. Therefore, it has been postulated that macrophages and microglia are the key cell types that are affected in HIV, while the neurons and glia cells are damaged by the factors released from the infected macrophages and microglia in the infected brain. HIV-encoded proteins and host factors from the macrophage and glial cells may mutually influence the function and fates of neurons. Based on studies using an animal model, it is thought that HIV can enter the brain early after systemic infection. Although the CNS is physiologically separated by the blood-brain barrier (BBB), the mechanism of action of the BBB in HIV-infected brains and the critical factors that lead to the development of HIV encephalopathy remain unclear.

Several chemokines and their receptors have been the focus of the studies on the pathogenesis of HIV encephalopathy.[7] It has been reported that CXCL8 (IL-8), CXCL10 (IP10), CXCL12 (SDF-1α, β), CCL2 (MCP1), CCL3 (MIP1α), CCL4 (MIP1β), CCL5 (RANTES), CCL7 (MCP3), and CX3CL1 (Fractalkine) are involved in the development of HIV encephalopathy. CXCR1, CXCR2, CXCR3, CXCR4, CCR1, CCR2, CCR3, CCR5, and CX3CR1 are known to be expressed in the CNS and might have a various role in maintaining the balance between neuroprotection and neurodegeneration.

The infection of perivascular macrophages and microglia may cause a disruption in the normal neurological functions either by producing viral proteins, such as gp120,[8] Tat, and Vpr,[9] or by exerting an indirect or bystander effect via some neurotoxic factors.[10–12] In addition, it has been proposed that after the inflammatory process, due to the establishment of a self-sustaining chain reaction, viral infection might play a more limited role in the degenerative process. Although both these mechanisms are not mutually exclusive and might coexist, the bystander theory is probably more consistent with most of the evidence (Fig. 2).

Recently, a hypothesis that the CXCR4-using X4 virus emitted to the neurons has been suggested.[13] Although the X4 virus may act as a neurotoxic factor in vitro thus far, there is little evidence indicating that the X4 virus plays a critical role in the human CNS. HIV-1 has certain characteristics that differ from other simian-related viruses, namely, simian immunodeficiency virus (SIV) and simian human immunodeficiency virus (SHIV): the latter is a recombinant virus that is obtained by replacing the SIV envelope with an HIV envelope. The X4 virus frequently infects the neurons in SIV- and SHIV-infected models.

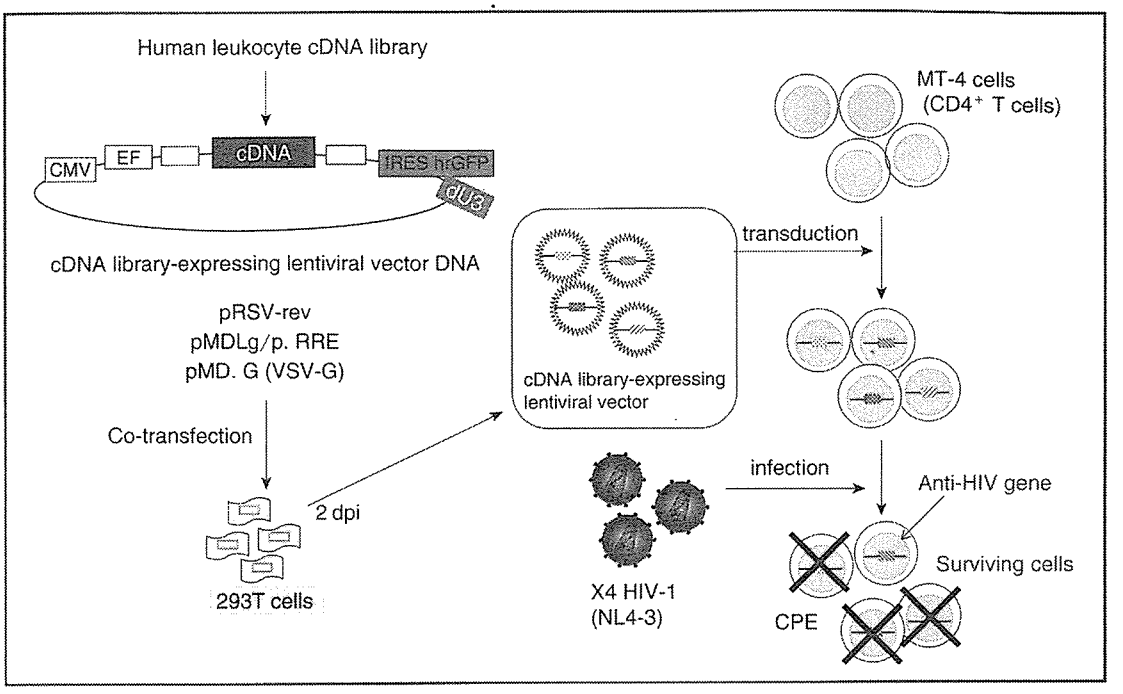


**Fig. 4 The screening system used for the detection of the anti-HIV gene**

A human leukocyte cDNA library was inserted into a vector and cotransfected with packaging plasmids into 293 T cells. The supernatant were transduced into MT-4 cells. They were infected with NL4-3 and the survivor cells were collected and analyzed.

By contrast, in the case of HIV-1, only the viruses using CCR5 have been found in the infected brain.

## New Models of HIV Encephalopathy

We have developed an animal model of HIV encephalopathy. We used the NOD-SCID mouse, which represented severe immunodeficiency acquired through heredity, and produces human chimeric mice by the intraperitoneal transplantation of human peripheral blood mononuclear cells (PBMCs). HIV-1, which uses CCR5, was then inoculated intraperitoneally. After establishing systemic HIV-1 infection, a bacterial component lipopolysaccharide was injected intraperitoneally (Fig. 3). Infiltration of human T cells and macrophages was induced in the mouse brain and many of these cells were found to be infected with HIV-1. Further, the astrocytes and microglia were activated. Importantly, the apoptosis of neurons was frequently detected near the human macrophages infected with HIV-1. On the other hand, using the X4 virus, we observed that significant neuronal death was not detected in the brain. The TRAIL molecule, which is one of the death-inducing ligands, was found to be predominantly expressed in the HIV-1-infected macrophages in the brain. When a neutralizing antibody against human TRAIL was injected intraperitoneally, neuronal apoptosis was significantly inhibited. This suggests that the TRAIL molecule is important for guiding the apoptosis of neurons in HIV encephalopathy.[12] Therefore, we propose that the TRAIL molecule may play a role in HIV encephalopathy. Our proposal was further confirmed by the examination of human pathological autopsy samples and cultured human neurons.[14,15]

Based on these results, we are focusing on the analysis of HIV pathogenesis in the CNS using small animals such as mice and rats. These animals are very useful in neuroscientific studies because a considerable amount of scientific knowledges has been amassed using these animals. Several experiments using the cells of these small animals have been reported in HIV research.[16]

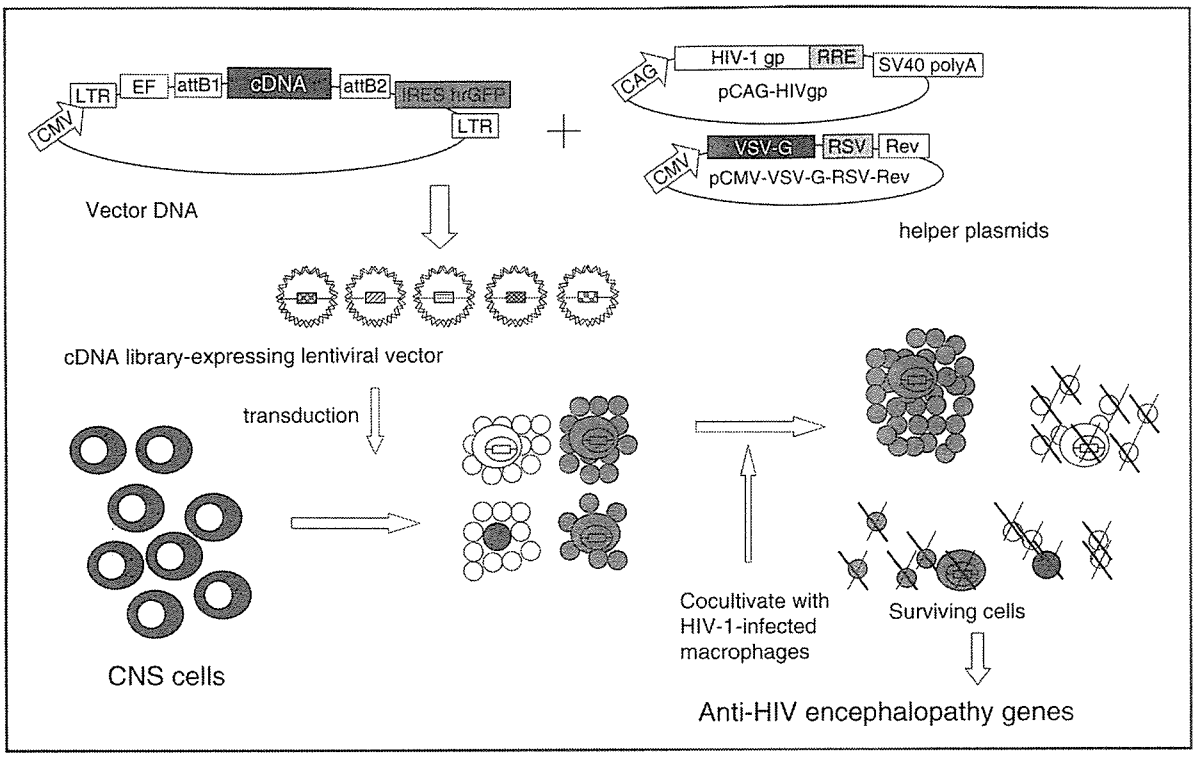


**Fig. 5 The analyzing system of anti-HIV encephalopathy genes**
The viral vectors expressing the rat brain cDNA were collected, transduced into the CNS cells, and cocultivated with HIV-1-infected macrophages. The surviving cells were then collected and analyzed.

Novel investigations have been carried out to determine the factors associated with this virus using a lentiviral vector system; this system was originally generated from HIV itself. We transduced the human leukocyte cDNA library into a human T cell line and then infected them with X4 HIV-1. After analyzing the gene that provides anti-HIV activity in the surviving cells, the CD14 gene[17] and an N-terminal deletion mutant of CD63 gene, namely CD63dN, were isolated (Fig. 4). CD14 appears to partially inhibit HIV-1 entry and provides resistance to HIV-1-induced cytopathic effect. CD63dN inhibits the surface expression of CXCR4. CD14 is one of the marker molecules of monocytes, and although the expression of CXCR4 can be seen in these cells, X4 virus cannot replicate efficiently in these cells. Further, since CD63 downregulates the activity of CXCR4 and the expression of CD63 is augmented in activated macrophages and microglia, it was suggested that CD63 might preferentially inhibit X4 HIV-1 infection in the infected brain. This explanation may be supported by the fact that it is difficult to detect X4 HIV-1 in the HIV-1-infected brain.

Further, we are trying to identify the genes that function against the cytopathic effect of HIV encephalopathy. An organic culture of the rat brains was cocultivated with the human macrophages with HIV infection. RNA was then collected from the samples of this culture, and was analyzed using a microarray system. Based on this analysis, the cDNA of a candidate gene was transduced into the rat brain cells and was cocultivated with HIV-infected human macrophages (Fig. 5). These experiments are currently in progress.

## Conclusion

To date, the research on HIV encephalopathy has been carried out by analyzing the brain tissues of clinical specimens and by infecting experimental animals with SIV or SHIV. In addition to these

models, a novel approach has been initiated using small animals such as mice and rats. Based on previous reports, it is thought that many host factors as well as viral factors are closely involved in the pathogenesis of the HIV encephalopathy, and the elucidation of its mechanisms is very important. Further, new types of researches that aim at identifying additional host factors have been initiated by using a lentiviral vector. They can be applied to devise powerful new medical treatments. Finally, it is important to improve these systems using small experimental animals and lentivirus for various central nervous system diseases.

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## References

1. Miura Y, Koyanagi Y. Death ligand-mediated apoptosis in HIV infection. Rev Med Virol. 2005;15:169–178.
2. Badley AD, Roumier T, Lum JJ, Kroemer G. Mitochondrion-mediated apoptosis in HIV-1 infection. Trends Pharmacol Sci. 2003;24:298–305.
3. Arnoult D, Petit F, Lelievre JD, Estaquier J. Mitochondria in HIV-1-induced apoptosis. Biochem Biophys Res Commun. 2003; 304:561–574.
4. McArthur JC. HIV dementia: an evolving disease. J Neuroimmunol. 2004;157:3–10.
5. Brew BJ. Evidence for a change in AIDS dementia complex in the era of highly active antiretroviral therapy and the possibility of new forms of AIDS dementia complex. AIDS. 2004;18 (Suppl 1):S75–78.
6. Koyanagi Y, Miles S, Mitsuyasu RT, Merrill JE, Vinters HV, Chen IS. Dual infection of the central nervous system by AIDS viruses with distinct cellular tropisms. Science. 1987;236:819–822.
7. Martin-Garcia J, Kolson DL, Gonzalez-Scarano F. Chemokine receptors in the brain: their role in HIV infection and pathogenesis. AIDS. 2002;16:1709–1730.
8. Kaul M, Lipton SA. Chemokines and activated macrophages in HIV gp120-induced neuronal apoptosis. Proc Natl Acad Sci USA. 1999;96:8212–8216.
9. Patel CA, Mukhtar M, Pomerantz RJ. Human immunodeficiency virus type 1 Vpr induces apoptosis in human neuronal cells. J Virol. 2000;74:9717–9726.
10. Achim CL, Heyes MP, Wiley CA. Quantitation of human immunodeficiency virus, immune activation factors, and quinolinic acid in AIDS brains. J Clin Invest. 1993;91:2769–2775.
11. Adamson DC, McArthur JC, Dawson TM, Dawson VL. Rate and severity of HIV-associated dementia (HAD): correlations with Gp41 and iNOS. Mol Med. 1999;5:98–109.
12. Miura Y, Misawa N, Kawano Y, et al. Tumor necrosis factor-related apoptosis-inducing ligand induces neuronal death in a murine model of HIV central nervous system infection. Proc Natl Acad Sci USA. 2003;100:2777–2782.
13. Zheng J, Thylin MR, Ghorpade A, et al. Intracellular CXCR4 signaling, neuronal apoptosis and neuropathogenic mechanisms of HIV-1-associated dementia. J Neuroimmunol. 1999; 98:185–200.
14. Miura Y, Koyanagi Y, Mizusawa H. TNF-related apoptosis-inducing ligand (TRAIL) induces neuronal apoptosis in HIV-encephalopathy. J Med Dent Sci. 2003;50:17–25.
15. Ryan LA, Peng H, Erichsen DA, et al. TNF-related apoptosis-inducing ligand mediates human neuronal apoptosis: links to HIV-1-associated dementia. J Neuroimmunol. 2004;148:127–139.
16. Persidsky Y, Limoges J, McComb R, et al. Human immunodeficiency virus encephalitis in SCID mice. Am J Pathol. 1996;149: 1027–1053.
17. Kawano Y, Yoshida T, Hieda K, Aoki J, Miyoshi H, Koyanagi Y. A lentiviral cDNA library employing lambda recombination used to clone an inhibitor of human immunodeficiency virus type 1-induced cell death. J Virol. 2004;78:11352–11359.

# Induction of Positive Cellular and Humoral Immune Responses by a Prime-Boost Vaccine Encoded with Simian Immunodeficiency Virus *gag/pol*[1]

**Kenji Someya,* Yasushi Ami,† Tadashi Nakasone,* Yasuyuki Izumi,* Kazuhiro Matsuo,* Shigeo Horibata,* Ke-Qin Xin,‡ Hiroshi Yamamoto,§ Kenji Okuda,‡ Naoki Yamamoto,* and Mitsuo Honda[2]***

**It is believed likely that immune responses are responsible for controlling viral load and infection. In this study, when macaques were primed with plasmid DNA encoding SIV *gag* and *pol* genes (SIV*gag/pol* DNA) and then boosted with replication-deficient vaccinia virus DIs recombinant expressing the same genes (rDIsSIV*gag/pol*), this prime-boost regimen generated higher levels of Gag-specific CD4$^+$ and CD8$^+$ T cell responses than did either SIV*gag/pol* DNA or rDIsSIV*gag/pol* alone. When the macaques were i.v. challenged with pathogenic simian/HIV, the prime-boost group maintained high CD4$^+$ T cell counts and reduced plasma viral loads up to 30 wk after viral challenge, whereas the rDIsSIV*gag/pol* group showed only a partial attenuation of the viral infection, and the group immunized with SIV*gag/pol* DNA alone showed none at all. The protection levels were better correlated with the levels of virus-specific T cell responses than the levels of neutralization Ab responses. These results demonstrate that a vaccine regimen that primes with DNA and then boosts with a replication-defective vaccinia virus DIs generates anti-SIV immunity, suggesting that it will be a promising vaccine regimen for HIV-1 vaccine development. *The Journal of Immunology,* 2006, 176: 1784–1795.**

The primary goals of any prophylactic HIV vaccine are to induce HIV-specific immune responses capable of preventing the malfunctioning of immune systems and to limit viral transmission due to replication. Clinical studies have demonstrated that CTL immune responses are associated with the reduction of plasma viral load (1, 2) and can control disease progression (3, 4). Replication of pathogenic SIV in vivo has also been shown to be controlled in the macaque model by CD8$^+$ T cell responses (5). Because amino acid sequences of Gag and Pol of HIV-1 proteins are relatively conserved, cross-clade and broad CTL responses targeting those proteins have been observed in both HIV-infected and HIV-exposed individuals, even if the latter group had not become infected (6–8). Thus, one recent focus of HIV vaccine research has been to elicit more protective antiviral immune responses by enhancing the expression levels of HIV-1 Ags of Gag and Pol using a safe vaccine vector.

Recently, several prime-boost regimens consisting of a DNA prime and a recombinant poxvirus boost targeting the immunodeficiency virus have been reported to generate higher levels of HIV-specific T cell immune responses than regimens relying on DNA or recombinant poxvirus vaccine alone (9, 10). In efficacy trials of such heterologous prime-boost vaccines, an SIV Ag encoding DNA prime and a boost of recombinant modified vaccinia virus Ankara (MVA)[3] elicited effective anti-SIV immunity and controlled infection of the nonpathogenic simian-HIV (SHIV) strain as well as of the pathogenic strain SHIV-89.6P in macaques (11–13) by effectively inducing CD8$^+$ CTL immunities. Various poxvirus vectors, i.e., an avipox virus, a canarypox virus, a fowlpox virus, a substrain of vaccinia Copenhagen (NYVAC), and MVA, have been evaluated for their usefulness, either alone or in combination with other vaccine modalities (14–18). To be useful, these vaccine vectors must, of course, be safe. The currently widely used MVA, which was developed toward the end of the campaign to eradicate small pox, has been effectively and safely used in >100,000 people as a small pox vaccine (19). MVA-based recombinant vector has also been reported to be safe in animals (20, 21). Lately, we have developed a replication-defective vaccinia virus DIs strain as a vaccine vector (22, 23). The DIs strain, generated by a 1-day-old egg passage of the DIE strain (24), has been proven safe (25, 26). We also suggested that a new prime-boost vaccine regimen consisting of SIV*gag/pol* DNA and rDIsSIV*gag/pol* might be useful for the development of an HIV-1 candidate vaccine that could induce strong cellular protective responses in mice (23). Lately, similar DNA/MVA vaccine combinations support the idea that the vaccine induced strong Ag-specific T and B cell responses (27). The prime-boost-vaccinated mice generated higher levels of both Gag-specific CD4$^+$ and CD8$^+$ T cell immune responses than those vaccinated with either DNA or rDIs alone. When such mice were challenged with SIV *gag/pol* expressing

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[3] Abbreviations used in this paper: MVA, modified vaccinia virus Ankara; rDIsSIV-*gag/pol*, recombinant DIs expressing SIV*gag* and *pol*; SFC, spot-forming cell; SHIV, simian-human immunodeficiency virus; SIV*gag/pol* DNA, plasmid DNA encoding SIV *gag* and *pol* genes; $TCID_{50}$, 50% tissue culture infectious doses.

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wild-type recombinant vaccinia virus, viral replication in the ovaries was controlled even in the absence of anti-DIs immunity. These results suggest that the new vaccine regimen, consisting of a DNA prime and a vaccinia virus DIs boost, safely and effectively elicits anti-immunodeficiency viral immunity.

In this study, we evaluated the vaccine efficacy of the prime-boost DNA/DIs vaccine encoding the *gag/pol* gene against a challenge with a highly pathogenic SHIV using 19 macaques. We hypothesize that the efficacy is mediated not only by the effect of virus-specific cellular immunity, but also by the effect of neutralization Ab responses against the challenged virus.

## Materials and Methods

### *Animals*

Nineteen female adult cynomolgus macaques (*Macaca fascicularis*) were purchased from Japan SLC. The macaques were fed and cared for in accordance with the standard operating procedure approved by the Ministry of Education, Culture, Sports, Science, and Technology of Japan. The study was performed in the P3 facility under guidelines established by the laboratory biosafety manual of the World Health Organization (28).

### *Preparation of vaccine Ags and challenge virus*

Plasmid DNA encoding SIV *gag* and *pol* genes (SIV*gag/pol* DNA) and recombinant DIs expressing the same genes (rDIsSIV*gag/pol*) were prepared as previously described (22, 23, 29). pcDNA3.1$^-$ and rDIsLacZ were used as controls of plasmid and recombinant viral Ags, respectively. After being immunized according to the protocol, animals were challenged with SHIV-C2/1 (30–32), which was a SHIV-89.6 variant isolated at the peak of initial plasma viremia from an infected cynomolgus macaque (31). The original SHIV strain was provided by Dr. Y. Lu (Harvard AIDS Institute, Cambridge, MA) (33, 34).

### *Enumeration of T PBL*

Fifty microliters of whole heparinized blood samples were stained with anti-human CD3 (clone HIT3a; BD Pharmingen), anti-human CD4 (clone SK3; BD Biosciences), and anti-human CD8 (clone SK1; BD Biosciences) for 15 min at 4°C. Blood samples were treated with FACS lysing solution for 15 min at 4°C, and then 50 $\mu$l of Flow Count (Beckman Coulter) was added. A FACSCalibur flow cytometer (BD Biosciences) was used to acquire 5000 CD3-positive, lymphocyte-gated events.

### *Intracellular IFN-γ cytokine staining*

Approximately $10^6$ of fresh PBMC were incubated with 0.2 $\mu$M pooled SIV Gag peptides spanning the full length of the Gag protein (AIDS Research and Reference Program, National Institutes of Health) together with 1 $\mu$g of anti-human CD28 (clone KOLT-2; Nichirei) and 1 $\mu$g of anti-human CD49d (clone 9F10; BD Pharmingen) in an appropriate volume of RPMI 1640 supplemented with 10% FBS and antibiotics for 16 h at 37°C. Then brefeldin A (Sigma-Aldrich) was added at 10 $\mu$g/ml, and the cells were incubated for an additional 4 h. After incubation, the cells were washed, stained with anti-human CD3 (clone HIT3a; BD Pharmingen) and anti-human CD8 (clone SK1; BD Biosciences) or anti-human CD4 (clone SK3; BD Biosciences) for 15 min. The cells were washed and then treated sequentially with FACS-lysing solution (BD Biosciences) and permeabliz-ing solution (BD Biosciences) for 10 min. The cells were stained with anti-human IFN-γ-FITC (clone 45.15; Immuno Tech) for 30 min and fixed with 2% paraformaldehyde solution. A FACSCalibur flow cytometer (BD Biosciences) was used to acquire 20,000 lymphocyte-gated events, which were then analyzed with CellQuest software (BD Biosciences).

### *Virus-specific IFN-γ ELISPOT assay*

An ELISPOT assay was performed following the method developed by Mothe and Watkins of the Wisconsin University Primate Center (35). Ninety-six-well, flat-bottom plates were coated with anti-IFN-γ mAb (clone MD-1; U-CyTech-BV) and blocked with 2% BSA in PBS. Fresh PBMC were added to the plate at $2 \times 10^5$ cells/well in triplicate and then incubated with 0.2 $\mu$M pooled SIV Gag peptides (AIDS Research and Reference Program) for 16 h at 37°C. Gold-labeled anti-biotin IgG solution (U-CyTech-BV) was added to the washed plates, which were then incubated for 1 h at 37°C. Individual spot-forming cells (SFC) were counted using the KS ELISPOT compact system (Zeiss) after a 15-min reaction with an activator mix (U-CyTech-BV). An SFC was defined as a large black spot with a fuzzy border (34).

### *Abs to SIV Gag p27 and SHIV 89.6P Env*

SIV Gag- and SHIV Env-specific IgG Ab end-point titers of the macaques' sera were measured by ELISA as previously described (23, 27, 30). All samples were run in triplicate at several dilutions. In brief, 96-well ELISA plates were coated with 0.3 $\mu$g of SIV p27 Gag (Advanced Biotechnologies) or 0.2 $\mu$M pooled SHIV 89.6P Env peptides (AIDS Research and Reference Program) per well. Heat-inactivated sera were serially diluted, then added to the ELISA plates. Gag- and Env-specific Abs bound to the Ags were captured with alkaline phosphatase-labeled goat anti-mouse IgG (EY Laboratories) and *p*-nitrophenyl-phosphate disodium substrate (Invitrogen Life Technologies).

The SHIV Env-specific neutralization Ab responses induced by challenge with SHIV were analyzed as previously described (28). In brief, 10 $\mu$g/ml purified macaque IgG was incubated with 100 50% tissue culture infectious doses ($TCID_{50}$) of SHIV-C2/1, then cultured in M8166 cells. The result was compared with parallel cultures to which preimmune IgG had been added. Neutralization was expressed as the percent inhibition of SIV Gag production in the culture supernatants. Anything >20% of inhibition was considered to be an efficient neutralization response.

### *Quantitation of plasma viral load*

Quantitation of SHIV genomic RNA copies in plasma samples was performed by real-time PCR with a TaqMan assay kit (PerkinElmer Applied Biosystems) and a PRISM 7700 sequence detection system (PerkinElmer Applied Biosystems) as previously described (30). Genomic RNA extracted from plasma samples and SIVmac239 (RNA standard; $5.4 \times 10^4$ RNA copies) were subjected to RT-PCR using an SIVmac239–1224 forward, SIVmac239–1326 reverse primer pair and an FAM-SIV-1272T probe. RNA copy numbers from plasma samples were calculated from the standard curve. Data were expressed as RNA copies per milliliter of plasma.

### *Flow cytometric detection of various subpopulations in CD4$^+$ T cells*

Approximately $10^6$ fresh PBMC were stained with anti-human CD4 (clone SK3; BD Biosciences), anti-human CD29 (clone 4B4; Beckman Coulter), and anti-human CD45RA (clone 5H9; BD Pharmingen) or with anti-human CD4 (clone *ν*-TH/I; Nichirei) and anti-human CD28 (clone KOLT-2, Nichirei). A FACSCalibur (BD Biosciences) was used to acquire 10,000 lymphocyte-gated events, which were then analyzed with CellQuest software.

### *Statistical analysis*

Data are expressed as the mean ± SD. The data analysis was conducted using the StatView program (SAS Institute), and all reported $p$ values are two-sided. Comparisons between groups were performed using the Kruskal-Wallis $H$ test, followed by the Student-Newman-Keuls correction. Correlations between protection and immune levels were analyzed using Spearman's rank correlation test. A value of $p < 0.05$ was considered significant.

## Results

### *Immunization protocol*

Plasmid DNA and the recombinant vaccinia DIs viruses with the inserted *gag/pol* region of SIVmac239 were constructed as previously described (23). Southern blotting confirmed that all plasmids and viruses had the expected genomic structures, whereas Gag-specific Western blots verified the in vitro expression of SIV Gag protein in rDIsSIV*gag/pol*-infected chick embryo fibroblasts (data not shown). In this study we opted to use the three-injection regimen for DNA immunization. Because we found that both the three- and five-injection DNA immunization strategies resulted in similar levels of T cell immunities (23). A total of 19 cynomolgus macaques were divided into four groups (Fig. 1). Group 1 macaques (prime-boost group of five macaques numbered M1 to M5) received three i.m. injections (2.5 mg) of each type of SIV*gag/pol* DNA at 8-wk intervals, followed by two injections of $10^8$ PFU of rDIsSIV*gag/pol*. Group 2 macaques (DNA group of five macaques numbered M6, M7, and M14 through M16) received three i.m. injections of the same dose of each type of SIV*gag/pol* DNA at

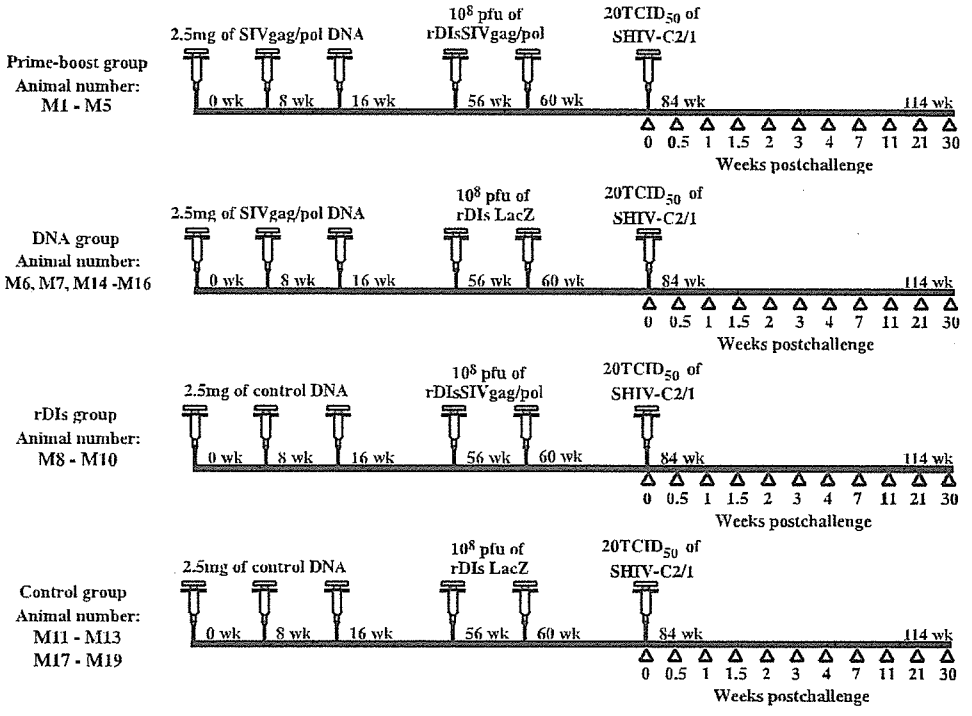


**FIGURE 1.** Scheme for immunization and viral challenge. Nineteen macaques were divided into four experimental groups and immunized with 2.5 mg of plasmid DNA at weeks 0, 8, and 16, then immunized with $10^8$ PFU of rDIs at weeks 56 and 60. Twenty-four weeks after the final immunization, macaques were challenged with 20 $TCID_{50}$ of SHIV.

8-wk intervals, followed by two injections of $10^8$ PFU of rDIsLacZ. Group 3 macaques (rDIs group of three macaques numbered M8 through M10) received three i.m. injections of control DNA pcDNA3.1$^-$ at 8-wk intervals, followed by two injections of $10^8$ PFU of rDIsSIV*gag/pol*. Group 4 (control group of six macaques numbered M11 through M13 and M17 through M19) received three i.m. injections of control DNA, followed by two injections of $10^8$ PFU of rDIsLacZ. Twenty-four weeks after the second booster inoculation, the macaques were i.v. challenged with 20 $TCID_{50}$ of pathogenic SHIV-C2/1, which were obtained by serum passages of SHIV-89.6. The effects of prime-boost vaccination with DNA and vaccinia DIs on protective immune induction were monitored for 30 wk, then animals were autopsied.

*Induction of cellular and humoral immune responses specific for SIV Gag*

We first analyzed the induction of cellular immunity by detecting the SIV Gag-specific IFN-γ ELISPOT activities of macaque PBMC after the first and third DNA primings and the first boosting of recombinant DIs in each animal (Fig. 2). A regimen of three consecutive immunizations with SIV*gag/pol* DNA induced 3- to 4-fold higher IFN-γ SFC than did a single immunization in the prime-boost and DNA groups ($p < 0.05$; Fig. 2, *A* and *B*). The numbers of IFN-γ-producing SFC increased ~8- to 9-fold after booster immunization with rDIsSIV*gag/pol* in the prime-boost group ($p < 0.01$; Fig. 2*C*). In contrast, no such increase was seen after booster immunization with rDIsLacZ in the DNA group (Fig. 2*C*). Macaques immunized with control DNA followed by rDIsSIV*gag/pol* (rDIs group) generated higher IFN-γ SFC than the DNA group ($p < 0.01$; Fig. 2, *B* and *C*). At no point in the course of immunization was Gag-specific IFN-γ SFC detected in the control group. Collectively, our findings show that the DNA/rDIs prime-boost immunization efficiently induced immunodeficiency virus-specific ELISPOT activity in macaques.

To substantiate the induction of cellular immunity specific for SIV Gag, intracellular IFN-γ staining was performed using PBMC after the first booster immunization with rDIs (Fig. 3). Of the four groups tested, the prime-boost group showed the highest frequency of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells. The frequencies of Gag-specific IFN-γ-producing $CD8^+$ and $CD4^+$ T cell responses to Gag peptides in the prime-boost group ranged from 0.51 to 1.22% with an average of 0.82%, and from 0.37 to 0.63% with an average of 0.46%, respectively. The expression of IFN-γ-producing $CD8^+$ and $CD4^+$ T cells immunized with either SIV*gag/pol* DNA (average of $CD8^+$ T cells, 0.095%; average of $CD4^+$ T cells, 0.015%) or rDIsSIV*gag/pol* (average of $CD8^+$ T cells, 0.27%; average of $CD4^+$ T cells, 0.05%) was apparently weak (Fig. 3). Therefore, as observed for the induction of the SIV Gag-specific ELISPOT activities, the prime-boost group proved to be the most efficient of the four animal groups tested at inducing Ag-specific intracellular IFN-γ cytokine staining.

To test for the induction of humoral immunity, we assessed the SIV Gag-specific IgG titers in the animals of each group (Fig. 4). Despite the elevation of Ab titers after the first immunization with SIV*gag/pol* DNA, no enhanced responses were observed after two serial immunizations with the DNA (Fig. 4*A*). However, although the titers did not exceed 2000, enhanced Ab responses were observed after booster immunization with rDIsSIV*gag/pol*. In summary, these results show that the prime-boost vaccine with DNA/rDIs predominantly elicits SIV Gag-specific cellular immune responses in immunized animals.

*Enhancement of SIV-specific T cell and humoral immune responses after viral challenge*

Twenty-four weeks after the second immunization with rDIs, macaques were challenged with highly pathogenic SHIV. As shown in Fig. 5*A*, Gag-specific IFN-γ SFC levels decreased on the day of challenge in all vaccinated groups, but the increase observed in the numbers of the SFC after SHIV challenge varied among the groups. The most pronounced increase was seen in the prime-boost group, with the average number of Gag-specific IFN-γ-producing cells increasing from 288/million PBMC on the day of challenge to 1124 ($p < 0.01$) 3 days after challenge. The DNA group increased from an average of 104 to 282 ($p < 0.01$), and the rDIs group from 114 to 347 ($p < 0.05$). No significant increases were noted in the control group.

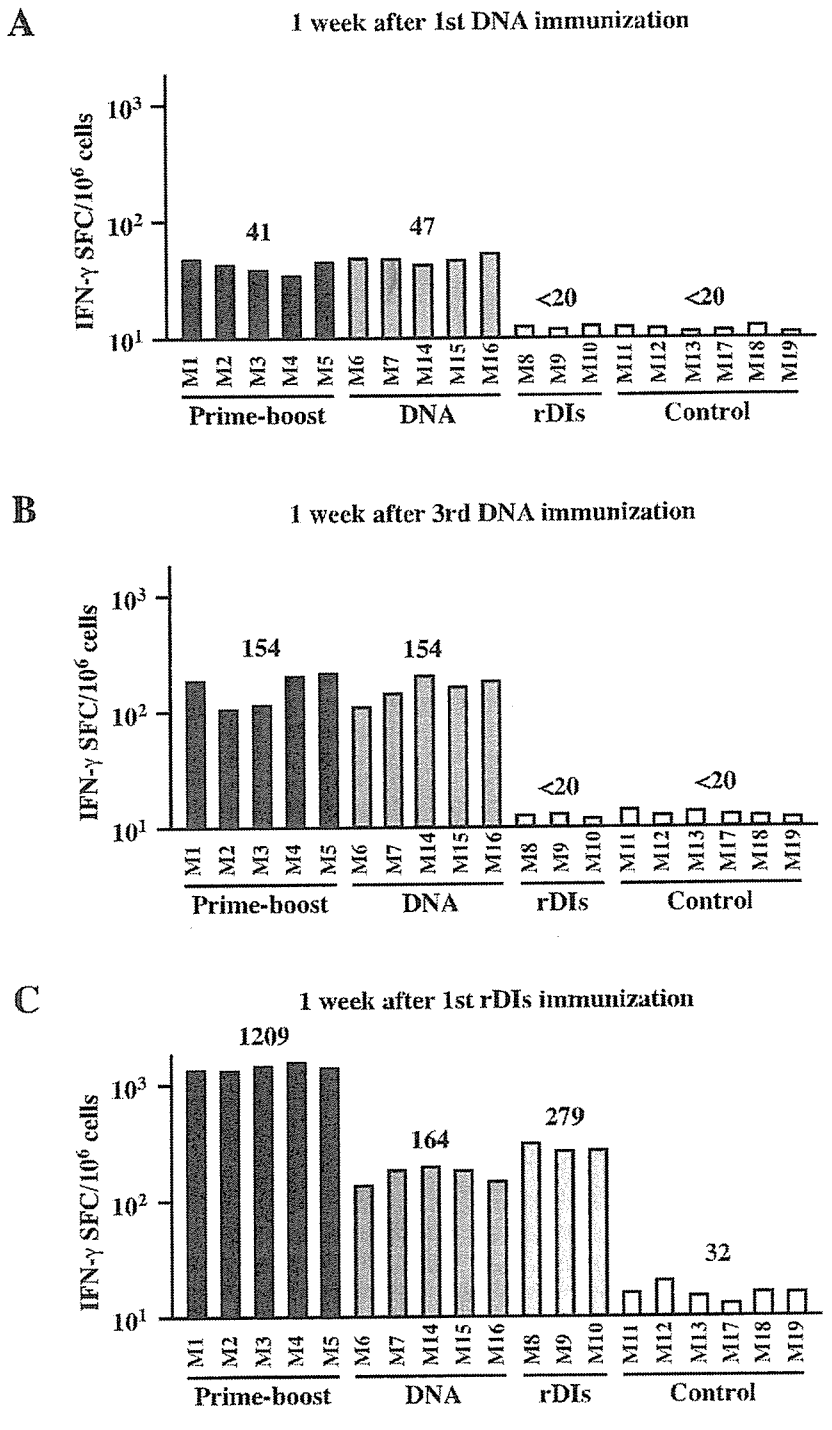


**FIGURE 2.** Frequency of SIV Gag-specific IFN-γ-producing cells in immunized macaques. Values are provided at 1 wk after the first DNA immunization (*A*), 1 wk after the third DNA immunization (*B*), and 1 wk after rDIs immunization (*C*). The numbers above the data bars represent the geometric means of the SFC levels in each group. Experimental groups and animal numbers are indicated at the bottom of the graph.

Intracellular IFN-γ staining of $CD8^+$ and $CD4^+$ T cells was also performed to assess any enhancement in immunodeficiency virus-specific immune responses (Fig. 5*B*). On the day of challenge, populations of Gag-specific IFN-γ-producing $CD8^+$ T cells in the prime-boost group averaged 0.32%, and populations of $CD4^+$ T cells averaged 0.11%. Three days after viral challenge, the average for Ag-specific IFN-γ-producing $CD8^+$ T cells rose to 0.61%, and that for $CD4^+$ T cells to 0.38%. Gag-specific IFN-γ-producing $CD8^+$ T cells averaged 0.18% for the DNA group and 0.25% for the rDIs group on the day of challenge, with those averages rising to 0.28 and 0.39%, respectively, by 3 days after challenge. Furthermore, the averages for Gag-specific $CD4^+$ T cells in the DNA and rDIs groups rose from 0.08 and 0.10 to 0.14 and 0.23%, respectively. The number of Ag-specific IFN-γ-producing $CD8^+$ and $CD4^+$ T cells in the control group was not affected by viral challenge. Thus, compared with the other three groups of animals, the prime-boost group showed the most significant enhancement of Ag-specific cellular immune responses after viral challenge, suggesting that Gag-specific memory T cell responses may be efficiently generated in animals by immunization with the prime-boost vaccine regimen.

To test the kinetics of humoral immune responses after SHIV challenge, we measured serum IgG titers to SIV Gag and SHIV 89.6P Env in all animals of each group (Fig. 4, *A* and *B*). The SIV Gag-specific IgG titers in all vaccinated animals were rapidly elevated and reached peak levels within 4 wk after challenge (Fig. 4*A*). The peak IgG titers in the prime-boost, DNA, and rDIs groups averaged 14,520 ± 2,508, 5,240 ± 1,099, and 8,400 ± 1,114,

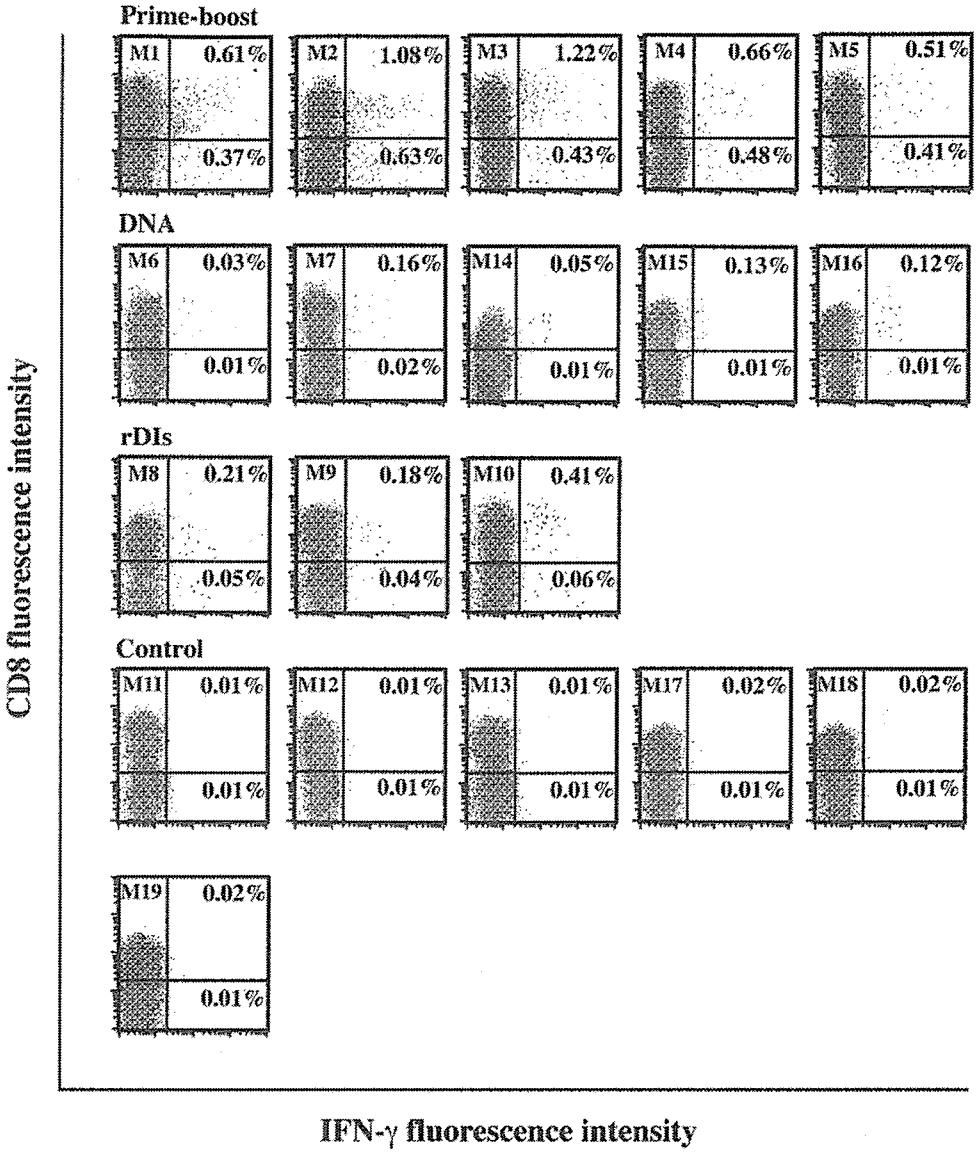


**FIGURE 3.** Flow cytometric analysis of SIV Gag-specific IFN-γ-producing $CD8^+$ T cells. One week after rDIs boost immunization, freshly isolated PBMC were stimulated with 0.2 μM pooled SIV Gag peptides and stained for anti-CD3, -CD8, and -IFN-γ. Twenty thousand lymphocyte-gated events were acquired. Upper and lower quadrants represent the frequencies of $CD8^+$ IFN-$\gamma^+$ and $CD4^+$ IFN-$\gamma^+$ T cells, respectively. Numbers indicate the percentage of cells in each quadrant.

respectively, with the increase in the prime-boost group reaching statistical significance ($p < 0.01$), compared with that in the DNA and rDIs groups. The Env-specific IgG appeared by 4 wk after challenge and reached peak levels between 7 and 11 wk. The peak IgG titers in the prime-boost, DNA, and rDIs groups averaged 5,200 ± 1,839, 3,180 ± 701, and 4,533 ± 833, respectively. Both the SIV Gag- and Env-specific IgG titers in the three vaccinated groups maintained high levels and persisted throughout the challenge period. In contrast, no IgG response to Gag and Env was detected in the control group. High titers of Env-specific IgG, but only very low levels of neutralization Ab responses to SHIV-C2/1, were induced in the DNA- and rDI-vaccinated groups (Fig. 4*C*). In contrast, the prime-boost macaques, especially M1, had high levels of neutralization Ab responses (viral neutralization >70%). Thus, these results show that the prime-boost vaccine with DNA/rDIs predominantly elicits SIV Gag-specific humoral responses in immunized animals and generates SHIV Env-specific binding and neutralization Abs after challenge with SHIV.

*Macaques of the prime-boost group control plasma viral load and block $CD4^+$-T cell depletion*

As noted above, the five macaques in the prime-boost group developed Ag-specific positive immunity after viral challenge. In these macaques, plasma viral loads were most attenuated and $CD4^+$ T cell counts best maintained in peripheral blood (Fig. 6). Peak viral loads occurred 2 wk after challenge in each group. The geometric means of the viral RNA copies were $1.1 \times 10^7$ copies in the prime-boost group, $4.7 \times 10^7$ copies in the DNA group, $4.1 \times 10^7$ copies in the rDIs group, and $4.5 \times 10^7$ copies in the control group (Fig. 6*A*). The difference observed in geometric mean peak viremia for the prime-boost and rDIs groups was significant ($p < 0.05$). Levels of peak viremia in the rDIs and control groups did not significantly differ. The peak viral loads in each had decreased by 7 wk after challenge, and the geometric means of the viral RNA copies from 7 to 30 wk were $8.1 \times 10^3$ copies (ranging from $7.1 \times 10^3$ to $9.4 \times 10^3$ copies) in the prime-boost group, $1.1 \times 10^6$ copies (ranging from $2.5 \times 10^5$ to $6.6 \times 10^6$ copies) in the DNA group, $7 \times 10^4$ copies (ranging from $5.3 \times 10^4$ to $1.1 \times 10^5$ copies) in the rDIs group, and $6.8 \times 10^6$ copies (ranging from $2.0 \times 10^6$ to $5.2 \times 10^7$ copies) in the control group (Fig. 6*A*). From 7 to 30 wk, the differences in the geometric means of the viral RNA copies between prime-boost and DNA groups ($p < 0.01$), prime-boost and rDIs groups ($p < 0.01$), and DNA and rDIs groups ($p < 0.01$) vs DNA and control groups ($p < 0.05$) were significant.

Two weeks after challenge, both DNA and control groups showed a serious depletion of $CD4^+$ T cells (to <50 cells) and a corresponding increase in viral RNA. In contrast, the prime-boost

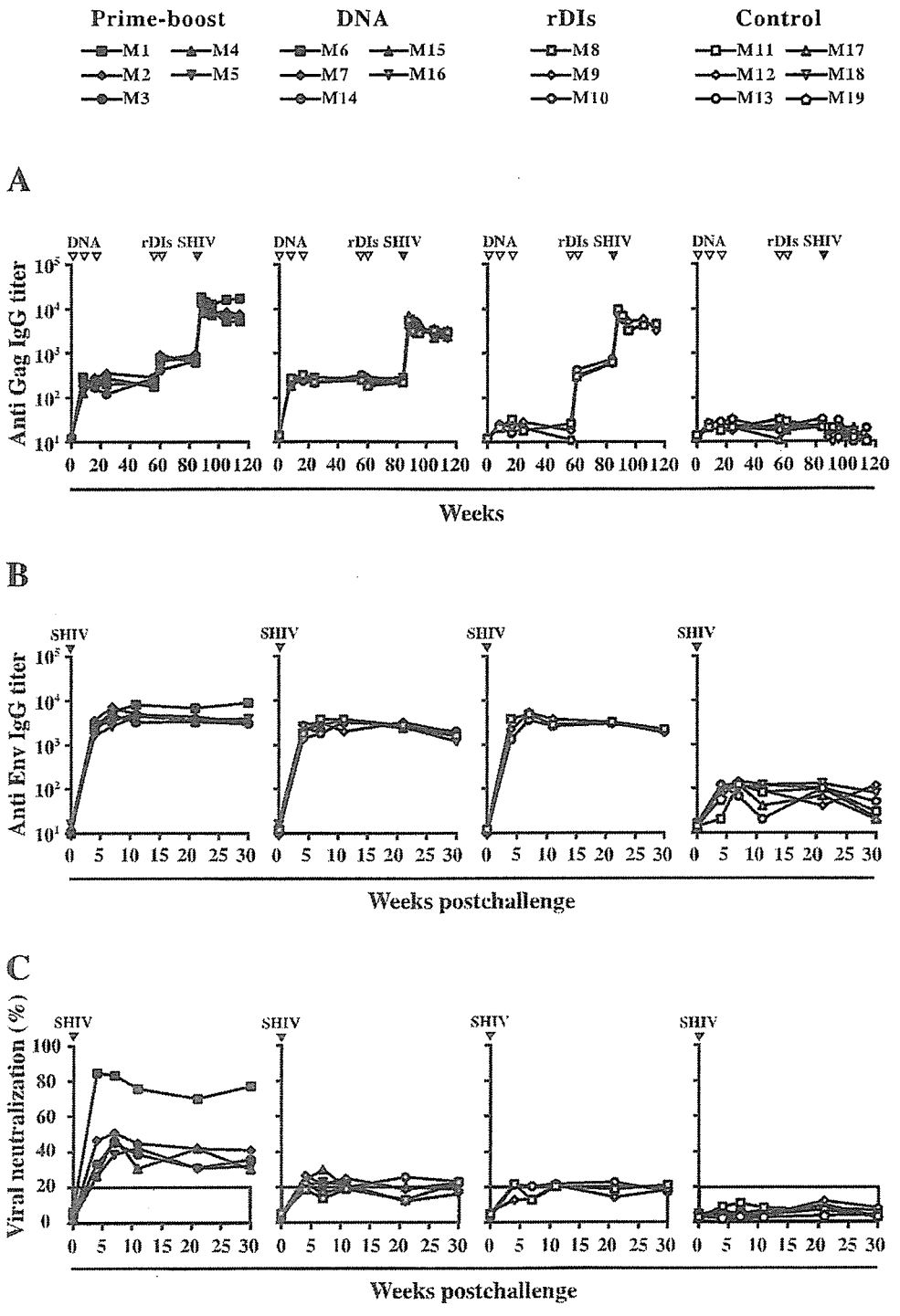


**FIGURE 4.** Kinetics of serum IgG titers specific to SIV Gag and SHIV 89.6P Env. *A*, SIV p27 Gag- and Env-specific IgG titers after immunization and after challenge. *B*, SHIV 89.6P Env-specific IgG titers. *C*, SHIV-specific neutralization responses. End-point titers of Gag- and Env-specific serum IgG and the percentage of SHIV-specific neutralization responses were measured at each time point. Results represent the average titer and percentage of the average viral neutralization value.

group maintained its $CD4^+$ T cell counts up to 30 wk after challenge (Fig. 6*B*). Four of the five macaques (M2–5) in the prime-boost group exhibited a gradual decrease in $CD4^+$ T cell counts; however, the macaques maintained an average of 254–303 cells from 2 to 30 wk after challenge. The one remaining macaque in the group (M1) maintained an average of 833 $CD4^+$ T cells (ranging from 630 to 1230 cells) and exhibited levels of viral RNA (<500 copies) that were undetectable except when peak viremia was reached at 2 wk ($5.7 \times 10^7$ copies) and transient viral replication occurred at 7 wk ($1.5 \times 10^4$ copies; Fig. 6, *A* and *B*).

To characterize the changes in the $CD4^+$ T cell subset in peripheral blood of each group after SHIV challenge, we used flow cytometric analysis to obtain an absolute count and to distinguish among the $CD29^+$, $CD45^+$, and $CD28^+$ cell subpopulations (Fig. 6, *C–E*). By 2 wk after challenge, a sharp decrease in the $CD29^+$ subset of $CD4^+$ T cells was seen in the DNA, rDIs and control groups (Fig. 6*C*). From 2 to 30 wk after challenge, the average number of this subset of cells in the DNA, rDIs, and control groups was 1.21% (ranging from 0.79 to 2.01%), 2.14% (ranging from 1.80 to 3.59%), and 1.03% (ranging from 0.55 to 1.89%), respectively. Similarly, the $CD45RA^+$ subset of $CD4^+$ T cells in the three groups rapidly declined by 2 wk after challenge, with the average of naive cells from 2 to 30 wk being 1.04% (ranging from 0.72 to 1.32%) in the DNA group, 2.83% (ranging from 1.04 to 4.78%) in the rDIs group, and 0.88% (ranging from 0.34 to 1.34%) in the control group (Fig. 6*D*). In contrast, the prime-boost group maintained the highest frequencies of both the $CD29^+$ subset, ranging from 8.0 to 9.63% with an average of 8.82% (Fig. 6*C*), and

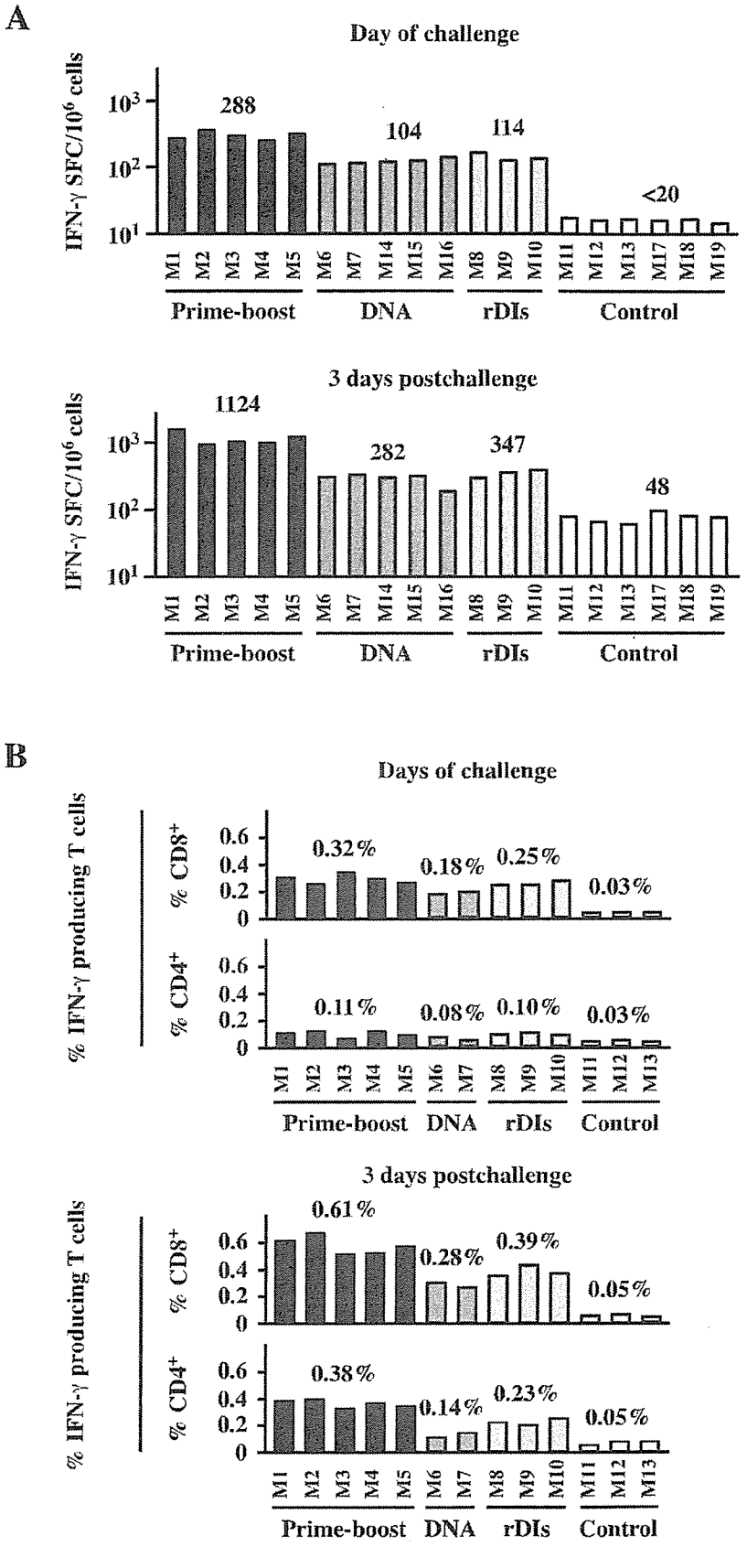


**FIGURE 5.** Comparison of IFN-γ ELISPOT activity and intracellular IFN-γ-producing T cells specific for SIV Gag in PBMC before and after viral challenge. *A*, ELISPOT activity. The numbers above the data bars represent the geometric means of SFC levels in each group. *B*, Intracellular IFN-γ-producing T cells. On the day of SHIV challenge and 3 days after SHIV challenge, freshly isolated cells were stimulated with SIV Gag peptides and stained for CD3, CD8, and IFN-γ. Numbers represent the percent average of the $CD4^+$ and $CD8^+$ T cell frequencies.

the $CD45^+$ subset, ranging from 6.29 to 9.16% with an average of 7.59% (Fig. 6, *C* and *D*). Flow cytometric analyses also revealed that the number of $CD4^+$ T cells expressing the costimulatory molecule CD28 rapidly dropped in the DNA, rDIs, and control groups by 2 wk after challenge (Fig. 6*E*). The average of $CD4^+CD28^+$ T cells from 2 to 30 wk after challenge in the DNA, rDIs, and control groups was 0.74% (ranging from 0.23 to 1.12%), 1.66% (ranging from 1.01 to 2.6%), and 0.91% (ranging from 0.61 to 1.13%), respectively. In contrast, $CD28^+CD4^+$ T cells in the prime-boost group ranged from 5.27 to 7.26%, with an average of

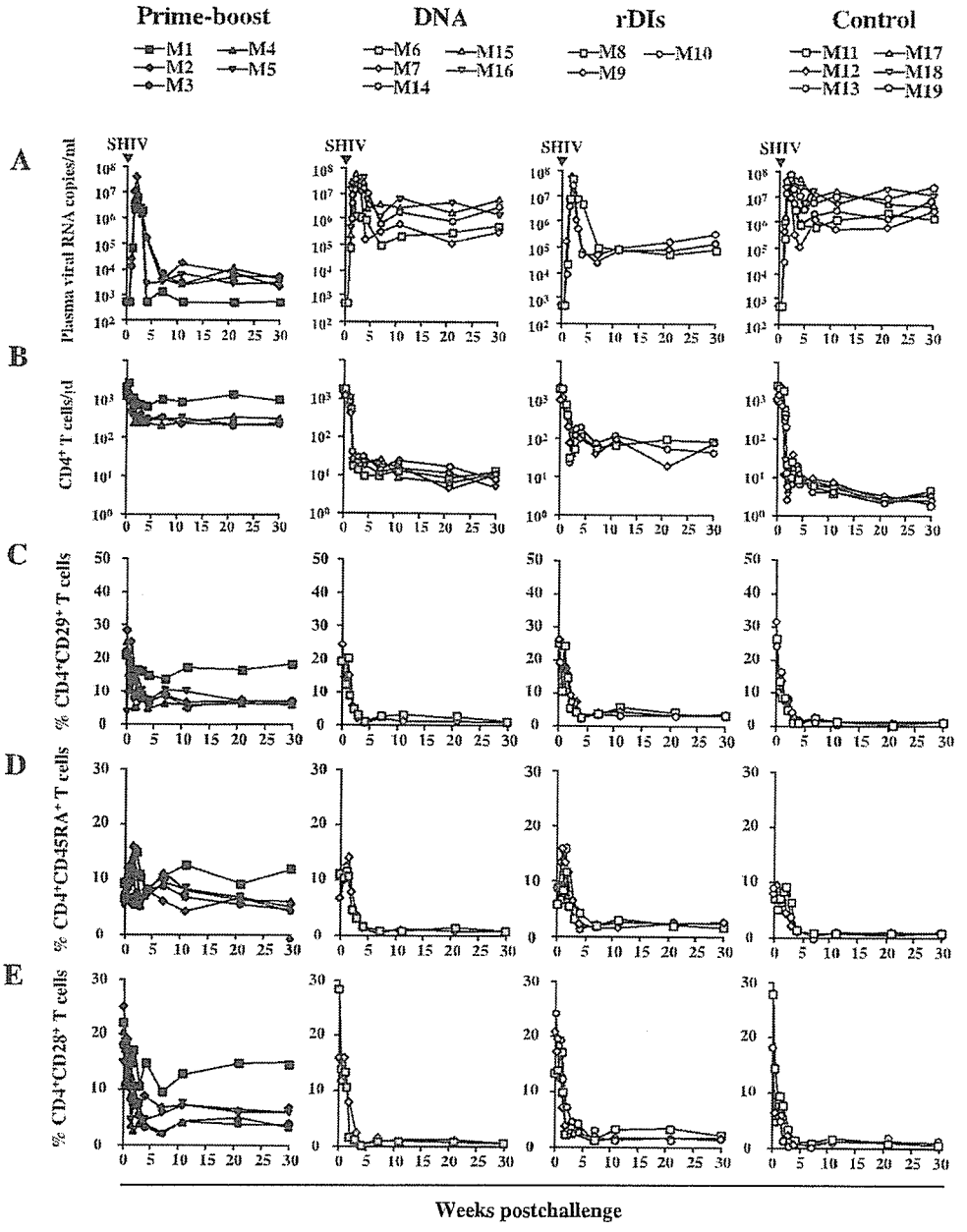


**FIGURE 6.** Kinetics of viral loads, $CD4^+$ T cell counts, and subpopulations of $CD4^+$ T cells in experimental groups after SHIV challenge. *A*, Plasma viral loads. Plasma viral loads were measured using the real-time PCR system. Levels <500 copies/ml were considered undetectable in this system. *B*, $CD4^+$ T cel counts. Whole blood was stained fo CD3, CD4, and CD8 Abs, and $CD4^+$ T cell counts were determined usin flow cytometry. *C*, $CD4^+CD29^+$ cells. *D*, $CD4^+CD45RA^+$ T cells. *E* $CD4^+CD28^+$ T cells. $CD4^+$ T ce subpopulations were not reduced the prime-boost animal group.

6.75%. Thus. the prime-boost group maintained $CD29^+$, $CD45RA^+$, and $CD28^+$ cell subpopulations in $CD4^+$ T cells after viral challenge.

*Controls of viremia and stability of $CD4^+$ blood lymphocytes correlate with Gag-specific IFN-γ SFC and neutralization Ab responses*

Because positive immune responses were detected in the animals immunized with the prime-boost vaccine of DNA/vaccinia DIs, we examined whether any immune responses correlated with the positive immunities using Spearman's rank correlation test (Fig. 7). The set-point levels of plasma viral RNA and $CD4^+$ T cell counts 7 wk after challenge significantly correlated with the Gag-specific IFN-γ SFC levels 3 days after challenge (plasma viral RNA levels vs Gag-specific IFN-γ SFC levels: Rs = 0.850, $p = 4.07 \times 10^{-6}$; $CD4^+$ T cell counts vs Gag-specific IFN-γ SFC levels: Rs = 0.968, $p = 1.10 \times 10^{-11}$; Fig. 7*A*). Interestingly, there was less correlation between the same set-point plasma viral RNA levels and $CD4^+$ T cell counts and the neutralization Ab responses 7 wk after challenge (plasma viral RNA levels vs percent viral neutralization: Rs = 0.796, $p = 4.53 \times 10^{-5}$; $CD4^+$ T cell counts vs percent viral neutralization: Rs = 0.851, $p = 3.93 \times 10^{-6}$). No correlation at all was observed between positive immune responses and anti-Gag and anti-Env Ab titers (data not shown).

## Discussion

It is believed likely that HIV-specific immune responses are associated with a decline in viral load and $CD4^+$ T cell maintenance. Our current study using the macaque model suggests that the prime-boost regimen, that is, priming with SIV*gag/pol* DNA followed by boosting with rDIsSIV*gag/pol*, modifies pathogenic SHIV infection. Furthermore, when the relationship between protection and the levels of immune responses was analyzed, we found that Gag-specific IFN-γ T cells showed a strong correlation and neutralization responses a weaker correlation with the suppression of plasma viral RNA levels and maintenance of $CD4^+$ T cell counts. These results accord with previous reports associating

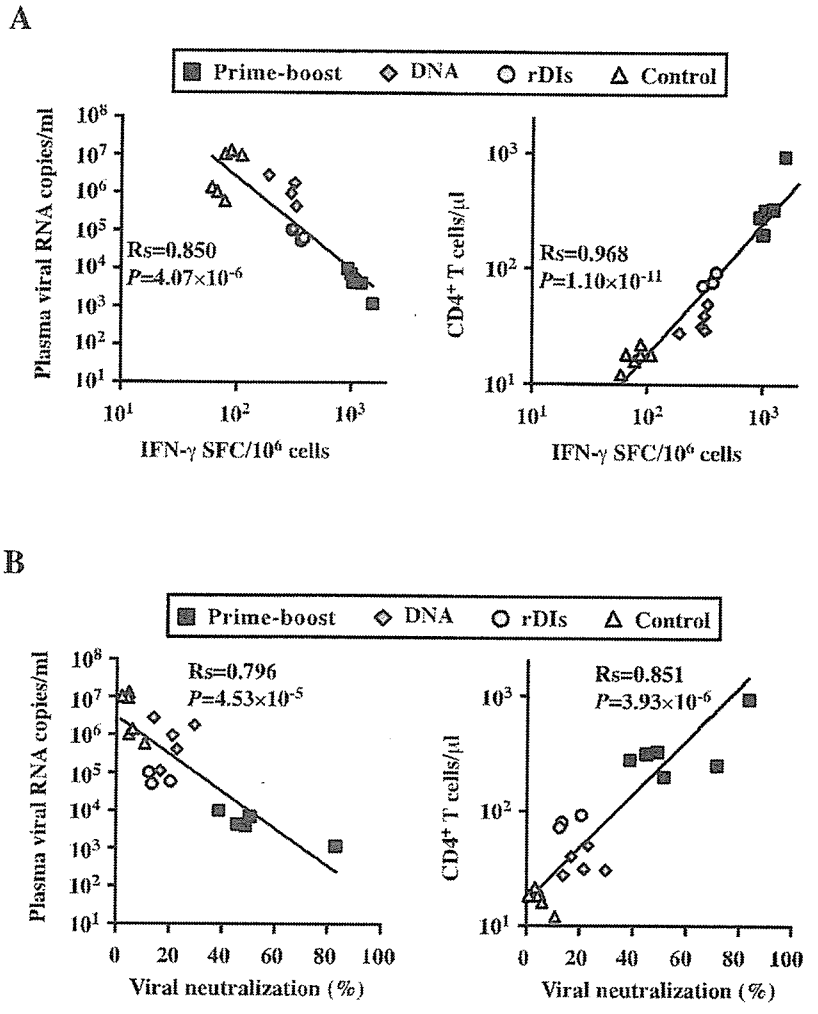


**FIGURE 7.** Correlations between protection and immune responses. *A*, The correlations between the decline in plasma viral RNA and the increased number of Gag-specific IFN-γ SFC, and between $CD4^+$ T cell counts and Gag-specific IFN-γ SFC. *B*, Correlations between the decline in plasma viral RNA and increased neutralization Ab responses, and between $CD4^+$ T cell counts and neutralization Ab responses. Correlations are calculated using Spearman's rank correlation test.

viral control with cellular immune responses in animals immunized with a prime-boost vaccination of either DNA/MVA (11) or cytokine-augmented DNA (36) encoding *gag* and *env* genes, followed by SHIV challenge. Neutralization Ab production was also detected in the animals (11, 36). Our new observations in vaccine research include the following: 1) Because positive immune responses better correlated with T cell than neutralization responses, it is probable that control of the plasma viral load and $CD4^+$ cell counts was achieved by virus-specific cellular immune responses. 2) Although our vaccine target was only Gag in this strategy, neutralization titers were detected in the prime-boost group that were higher than those induced in animals immunized with DNA, rDIs, or vector controls alone. These higher titers of the neutralization Ab responses against challenge virus might account for the presence of a high number of $CD4^+$ T cells in prime-boost animals (Fig. 6*B*) and might be associated with the production of neutralization Ab. It may, therefore, be reasonable to conclude that anti-Env neutralization Abs were effectively induced in the animals after SHIV challenge. Thus, we suggest that not only cellular responses, but also neutralizing Ab responses, elicited by the challenge virus may play a role in the pathogenesis of HIV/AIDS in the macaque model. 3) This vaccination regimen consisted of DNA and a nonreplicating vaccinia virus DIs, which is very safe even in immunodeficient states. Although other highly attenuated vaccinia strains replicate under synchronized viral infections to mammalian cells (37, 38), the DIs does not replicate in any mammalian cells tested because of natural big deletion of the genome (22, 23, 39). Thus, DIs vaccination eliminates the risk of a disseminated or progressive vaccinia viral infection in the immunocompromised, HIV-infected individual. Therefore, the DNA/DIs vaccine will be most safe in mammalians and may be suitable for therapeutic vaccine.

Recently, we demonstrated that priming with SIV*gag/pol* DNA, followed by boosting with rDIsSIV*gag/pol* generated both Th1-type $CD8^+$ and $CD4^+$ T cell responses specific for SIV Gag, resulting in the protection of immunized mice from a wild-type vaccinia virus recombinant expressing SIV Gag and Pol (23). Our previous mouse and macaque results (23, 40) (Fig. 6) showed that DNA alone was not as effective at inducing positive immunity in the macaque AIDS model as had been reported by others (11, 36). This discrepancy may depend on differences in DNA preparation, for example, whether the target HIV DNA was optimized to the human codon.

Although the exact immune mechanism responsible for protection from viral infection is not yet fully understood, both Ag-specific $CD4^+$ and $CD8^+$ T cell responses were clearly enhanced by viral infection in the prime-boost-immunized animals that exhibited a pronounced attenuation of plasma viral load. Our finding that challenge with the highly pathogenic SHIV virus enhances cellular immunity confirms the results of a recent study (41). It has been demonstrated that HIV-specific $CD8^+$ T cell responses play an important role in controlling viral replication by cytolysis and cytokine and/or anti-virus factor production (1, 2, 3, 42, 43). Others have also documented that HIV-specific $CD4^+$ T cell responses contribute to virus control or the slowing of disease progression (44–46). The critical role played by $CD4^+$ T cell responses against viral infections was also reported in studies of murine

lymphatic choriomeningitis viral infection (47) and CMV immunity in bone marrow transplant recipients (48). DNA/poxvirus prime-boost vaccination induced a high frequency and a high avidity of $CD8^+$ cytotoxic T lymphocyte populations (49), with the magnitude of HIV/SIV-specific $CD4^+$ and/or $CD8^+$ T lymphocyte responses in the course of infection inversely correlating with the viral load (50, 51). In addition, MHC class I molecules loading CTL epitopes may help control viral replication (52–56). The exact mechanisms underlying protective immune responses against HIV-1 remain a subject of debate; however, the above studies suggest that the simultaneous induction after vaccination of both Ag-specific $CD8^+$ and $CD4^+$ T cell responses may make it possible to attenuate immunodeficient viral infection. In this study we showed the efficacy of the prime-boost vaccination by monitoring IFN-γ ELISPOT, intracellular IFN-γ, and Ab responses. In the prime-boost group, boosting with rDIsSIV*gag/pol* induced ELISPOT responses (average of 1209 SFC) almost 10-fold higher than those induced by SIV*gag/pol* DNA (average of 154 SFC). In addition, intracellular IFN-γ staining revealed that the prime-boost vaccination generated high levels of Gag-specific intracellular IFN-γ-producing $CD8^+$ T cells (average, 0.82%; range, 0.51–1.22%) as well. However, lower Gag-specific T cell responses were observed in macaques vaccinated with either SIV*gag/pol* DNA or rDIsSIV-*gag/pol* alone than with the prime-boost regimen. In contrast to the strong Gag-specific T cell responses generated by the prime-boost vaccination, humoral responses specific for the same Ag were apparently low throughout the course of immunization. Although the peak IgG titers in the prime-boost group were observed after the first or second boosting with rDIsSIV*gag/pol*, Ab titers remained low. These results are in line with our previous study using the mouse model (23), suggesting that our prime-boost vaccine immunodominantly generates SIV Gag-specific cellular responses in macaques.

Monitoring ELISPOT and intracellular IFN-γ T cell responses specific for Gag revealed that responses decreased at the time of challenge with pathogenic SHIV, but then rapidly recovered. Gag-specific IFN-γ ELISPOT responses in the prime-boost group averaged 288 SFC on the day of challenge and increased to 1124 SFC on day 3 after challenge. The population of intracellular IFN-γ-producing $CD8^+$ and $CD4^+$ T cells specific for Gag also increased from an average of 0.32 to 0.61% and from an average of 0.11 to 0.38%, respectively, suggesting that our prime-boost vaccine generated a high frequency of very responsive $CD4^+$ and $CD8^+$ memory T cells that immediately reactivated sufficient levels of the Ag-specific immune responses against the SHIV Ag. Furthermore, a kinetic study of plasma viral loads and counts of $CD4^+$ T cells after challenge with SHIV revealed different patterns for each group. Although peak plasma viral loads were observed 2 wk after challenge in all groups, the number of plasma RNA copies peaking at that time in the prime-boost group were ~5-fold lower than in other groups, with numbers remaining depressed during the period extending from 7 to 30 wk after infection. However, high $CD4^+$ T cell counts were maintained in the prime-boost group. These results suggest a correlation between both plasma viral loads and the maintenance of high $CD4^+$ T cell counts and T cell response levels.

With regard to safety of vaccinia DIs as a vaccine vector, its viral replication occurs only in chick embryo fibroblasts, not in any mammalian cell lines tested (22, 24–26, 57). Because a vaccine regimen combining DNA and a defective DIs vector would not run the risk that the virus used as vector might replicate and disseminate, it would pose less of a risk to a severely immunocompromised host. Furthermore, in this study using the macaque model, we demonstrated that the cellular immune responses generated by the prime-boost vaccination were higher than those induced by vaccination with either DNA or rDIs alone and that response levels correlated to plasma viral loads and $CD4^+$ T cell counts after challenge with pathogenic SHIV. In summary, these results demonstrate that the new prime-boost regimen safely and effectively elicits anti-immunodeficiency viral immunity, suggesting its promise as a potential vaccine against HIV-1 infection as well as against HIV-induced disease progression.

Current macaque models of HIV, SIV, and SHIV may fall short of precisely mirroring human HIV infection. In some macaque HIV/AIDS models, SIVmac239 has been targeted as a desirable challenge virus, because it is a typical CCR5-tropic SIVmac and can cause both chronic and progressive disease in macaques (41, 58, 59). However, the virus is very difficult to neutralize and also very difficult to clear even from animals that have been previously immunized with homologous recombinant vector-based vaccines (41, 58, 59). Only live attenuated SIV has been reported to control SIVmac239 (T. Allen, Global HIV Vaccine Enterprise Meeting, Washington, October 21, 2004). Although there may be no macaque model suitable for evaluating the efficacy of an SIV or HIV experimental immunogen, in this study we clearly showed that vaccination with an SIV experimental immunogen consisting of SIV*gag/pol* DNA and replication-defective rDIsSIV*gag/pol* caused a pronounced attenuation of the infection caused by a highly pathogenic variant of SHIV-C2/1 in all five macaques tested. SHIV-C2/1, used as challenge virus, is a variant of SHIV 89.6. Because SHIV89.6 does not induce both a marked decline in $CD4^+$ cells and a high level of plasma viral load in cynomolgus macaques, we passaged serum from virus-infected cynomolgus macaques. The variant was obtained by the serum passages using cynomolgus macaques inoculated with SHIV89.6, and it induced high levels of viremia ($1–10 \times 10^7$ viral RNA copies/ml) and marked $CD4^+$ T cell depletion (<10 cells/μl) within 2 and 3 wk after viral inoculation (30, 31, 39). Furthermore, genomic study revealed 16 mutations of genomic DNA and 15 amino acid mutations in the Env region of parental virus. Thus, the cynomolgus AIDS model challenged with SHIV-C2/1 may represent primary HIV-1 infection in humans. These results should prove useful in determining how potent the new prime-boost vaccine regimen might be at eliciting anti-immunodeficiency virus immunity.

HIV-1 has been reported to preferentially infect $CD45RO^+CD4^+$ T cells in the early stages of infection, with the number of $CD45RA^+CD4^+$ T cells declining in later stages (60–62). Furthermore, the loss of this subpopulation of $CD4^+$ T cells during the early phase of immunodeficiency virus infection correlates to disease progression (63, 64), whereas the low $CD45RA^+CD4^+$ T cell levels in the late stages of infection correlate with an increased risk of death (65–67). The levels of $CD4^+$ T cells expressing the $CD28^+$ molecule have also been demonstrated to correlate with disease progression (68, 69). To confirm the effect of prime-boost immunity after SHIV challenge, we analyzed the kinetics of $CD4^+$ T cells expressing $CD29^+$, $CD45RA^+$, and $CD28^+$ molecules. We observed that the prime-boost group maintained the subpopulations of $CD4^+$ T cells throughout the course of infection, with an average of 8.82% $CD29^+$ cells, 7.59% $CD45\ RA^+$ cells, and 6.75% $CD28^+$ cells. In contrast, $CD4^+$ T cell populations in the other DNA and rDIs groups were reduced to <3%. These results suggest that immunization with the new prime-boost regimen induces protective immunity while maintaining the levels of the various $CD4^+$ T cell subpopulations.

In summary, our study has shown that the vaccine strategy that primes with DNA and then boosts with the replication-defective

vaccinia virus DIs generates both $CD4^+$ and $CD8^+$ T cell responses specific for SIV Gag, resulting in protection of the immunized macaques from pathogenic SHIV. However, it remains to be elucidated whether the *gag/pol*-encoding vaccine may elicit a protective effect against various viral challenges, such as CCR5-tropic viruses and other primary viruses. Nonetheless, this new regimen's twin merits of safety and efficacy position it as a promising vaccine candidate against HIV-1 infection as well as against HIV-induced disease progression.

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## Disclosures

The authors have no financial conflict of interest.

## References

1. 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.
2. Ogg, G. S., X. Jin, S. Bonhoeffer, P. R. Dunbar, M. A. Nowak, S. Monard, J. P. Segal, Y. Cao, S. L. Rowland-Jones, V. Cerundolo, et al. 1998. Quantitation of HIV-1 specific cytotoxic T lymphocytes and plasma load of viral RNA. *Science* 297: 2103–2106.
3. Ogg, G. S., S. Kostense, M. R. Klein, S. Jurriaans, D. Hamann, A. J. McMichael, and F. Miedema. 1999. Longitudinal phenotypic analysis of human immunodeficiency virus type 1-specific cytotoxic T lymphocytes: correlation with disease progression. *J. Virol.* 73: 9153–9160.
4. Wagner, R., B. Leschonsky, E. Harrer, C. Paulus, C. Weber, B. D. Walker, S. Buchbinder, H. Wolf, J. R. Kalden, and T. Harrer. 1999. Molecular and functional analysis of a CTL epitope in HIV-1 p24 recognized from a long-term nonprogressor: constraints on immune escape associated with targeting a sequence essential for viral replication. *J. Immunol.* 162: 3727–3734.
5. Jin, X., D. E. Bauer, S. E. Tuttleton, S. Lewin, A. Gettie, J. Blanchard, G. E. Irwin, J. T. Safrit, J. Mittler, L. Weinberger, et al. 1999. Dramatic rise in plasma viremia after $CD8^+$ T cell depletion in simian immunodeficiency virus infected macaques. *J. Exp. Med.* 189: 991–998.
6. Betts, M. R., J. Krowka, C. Santamaria, K. Balsamo, K. Gao, G. Mulundu, C. Luo, N. N'Gandu, H. Sheppard, B. H. Hahn, et al. 1997. Cross-clade human immunodeficiency virus (HIV)-specific cytotoxic T-lymphocytes responses in HIV-infected Zambians. *J. Virol.* 71: 8908–8911.
7. Durali, D., J. Morvan, F. Letourneur, D. Schmitt, N. Guegan, M. Dalod, S. Saragosti, D. Sicard, J. P. Levy, and E. Gomard. 1998. Cross-reactions between the cytotoxic T-lymphocytes responses of human immunodeficiency virus-infected African and European patients. *J. Virol.* 72: 3547–3553.
8. McAdam, S., P. Kaleebu, P. Krause, P. Goulder, N. French, B. Collin, T. Blanchard, J. Whitworth, A. McMichael, and F. Gotch. 1998. Cross-clade recognition of p55 by cytotoxic T lymphocytes in HIV-1 infection. *AIDS* 12: 571–579.
9. Kent, S. J., A. Zhao, S. J. Best, J. D. Chandler, D. B. Boyle, and I. A. Rhamsaw. 1998. Enhanced T-cell immunogenicity and protective efficacy of human immunodeficiency virus type 1 vaccine regimen consisting of consecutive priming with DNA and boosting with recombinant fowlpox virus. *J. Virol.* 72: 10180–10188.
10. Robinson, H. L., D. C. Montefiori, R. P. Johnson, K. H. Hanson, M. L. Kalish, J. D. Lifson, T. H. Rizvi, S. Lu, S. L. Hu, G. P. Mazzara, et al. 1999. Neutralizing antibody-independent containment of immunodeficiency virus challenge by DNA priming and recombinant poxvirus booster immunizations. *Nat. Med.* 5: 526–534.
11. Amara, R. R., F. Villinger, J. D. Altman, S. L. Lydy, S. P. O'Neil, S. I. Staprans, D. C. Montefiori, Y. Xu, J. G. Herndon, L. S. Wyatt, et al. 2001. Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. *Science* 296: 69–74.
12. Amara, R. R., F. Villinger, S. I. Staprans, J. D. Altman, D. C. Montefiori, N. L. Kozyr, Y. Xu, L. S. Wyatt, P. L. Earl, J. G. Herndon, et al. 2002. Different patterns of immune responses but similar control of a simian-human immunodeficiency virus 89.6P mucosal challenge by modified vaccinia virus Ankara (MVA) and DNA/MVA vaccines. *J. Virol.* 76: 7625–7631.
13. Tang, Y., F. Villinger, S. I. Staprans, R. R. Amara, J. M. Smith, J. G. Herndon, and H. L. Robinson. 2002. Slowly declining levels of viral RNA and DNA in DNA/recombinant modified vaccinia virus Ankara-vaccinated macaques with controlled simian-human immunodeficiency virus SHIV-89.6P challenge. *J. Virol.* 76: 10147–10154.
14. Radaelli, A., and C. De Giuli Morghen. 1994. Expression of HIV-1 envelope gene by recombinant avipox. *Vaccine* 12: 1101–1109.
15. Santra, S., J. E. Schmitz, M. J. Kuroda, M. A. Lifton, C. E. Nickerson, C. I. Lord, R. Pal, G. Franchini, and N. L. Letvin. 2002. Recombinant canarypox vaccine-elicited CTL specific for dominant and subdominant simian immunodeficiency virus epitopes in rhesus monkeys. *J. Immunol.* 168: 1847–1853.
16. Radaelli, A., C. Zanotto, G. Perletti, V. Elli, E. Vicenzi, G. Poli, and C. De Giuli Morghen. 2003. Comparative analysis of immune responses and cytokine profiles elicited in rabbits by the combined use of recombinant fowlpox viruses and virus-like particles in prime-boost vaccine protocols against SHIV. *Vaccine* 21: 2061–2073.
17. Hel, Z., J. Nacsa, W. P. Tsai, A. Thornton, L. Giuliani, J. Tartaglia, and G. Franchini. 2002. Equivalent immunogenicity of the highly attenuated poxvirus-based ALVAC-SIV and NYVAC-SIV vaccine candidate in SIVmac251-infected macaques. *Virology* 304: 125–134.
18. Hanke, T., R. V. Samuel, T. J. Blanchard, V. C. Neumann, T. M. Allen, J. E. Boyson, S. A. Sharpe, N. Cook, G. L. Smith, D. I. Watkins, et al. 1999. Effective induction of simian immunodeficiency virus-specific cytotoxic T lymphocytes in macaques by using a multiepitope gene and DNA prime-modified vaccinia virus Ankara boost vaccination regimen. *J. Virol.* 73: 7524–7532.
19. Mayr, A., H. Stickel, H. K. Muller, K. Danner, and H. Singer. 1978. The smallpox vaccination strain MVA: marker, genetic, structure, experience gained with the parenteral vaccination and behavior in organisms with a debilitated defense mechanism. *Zentralbl. Bakteriol. Ser. B* 167: 375–390.
20. Hanke, T., A. J. McMichael, R. V. Samuel, L. A. Powell, L. McLoughlin, S. J. Corme, and A. Edlin. 2002. Lack of toxicity and persistence in the mouse associated with administration of candidate DNA and modified vaccinia virus Ankara (MVA)-based HIV vaccine for Kenya. *Vaccine* 22: 108–114.
21. Ober, B. T., P. Bruhl, M. Schmidt, V. Wieser, W. Gritschenberger, S. Coulibaly, H. Savidis-Dacho, M. Gerencer, and F. G. Falkner. 2002. Immunogenicity and safety of defective vaccinia virus lister: comparison with modified vaccinia virus Ankara. *J. Virol.* 76: 7713–7723.
22. Ishii, K., Y. Ueda, K. Matsuo, Y. Matsuura, T. Kitamura, K. Kato, Y. Izumi, K. Someya, T. Ohsu, and M. Honda. 2002. Structure analysis of vaccinia virus DIs strain: application as a new replication-deficient viral vector. *Virology* 302: 433–444.
23. Someya, K., K. Q. Xin, K. Matsuo, K. Okuda, N. Yamamoto, and M. Honda. 2004. A consecutive prime-boost vaccination of mice with simian immunodeficiency virus (SHIV) gag/pol DNA and recombinant vaccinia virus strain DIs elicits anti-SIV immunity. *J. Virol.* 78: 9842–9853.
24. Tagaya, I., T. Kitamura, and Y. Sano. 1961. A new mutant of dermovaccinia virus. *Nature* 192: 381–382.
25. Tagaya, I., H. Amano, T. Kitamura, T. Komatsu, Y. Ueda, Y. Tanaka, N. Uchida, and H. Kodama. 1973. Properties of an attenuated mutant of vaccinia virus, strain DIs. *Symp. Ser. Immunobiol. Stand.* 19: 299–307.
26. Tagaya, I., H. Amano, T. Komatsu, N. Uchida, and H. Kodama. 1974. Supplement to the pathogenicity and immunogenicity of an attenuated vaccinia virus, strain DIs, in cynomolgus monkeys. *Jpn J. Med. Sci. Biol.* 27: 215–228.
27. Hutchings, C. L., S. C. Gilbert, A. V. S. Hill, and A. C. Moore. 2005. Novel protein and poxvirus-based vaccine combinations for simultaneous induction of humoral and cell-mediated immunity. *J. Immunol.* 175: 599–606.
28. Someya, K., D. Cecilia, T. Nakasone, Y. Ami, K. Matsuo, S. Burda, H. Yamamoto, N. Yoshino, M. Kaizu, S. Ando, et al. 2005. Vaccination of rhesus macaques with recombinant *Mycobacterium bovis* bacillus Calmette-Guérin (BCG)-Env V3 elicits neutralizing antibody-mediated protection against simian-human immunodeficiency virus with a homologous but not a heterologous V3 motif. *J. Virol.* 79: 1452–1462.
29. Sasaki, S., K. Sumino, K. Hamajima, J. Fukushima, N. Ishii, S. Kawamoto, H. Mohri, C. R. Kensil, and K. Okuda. 1998. Induction of systemic and mucosal immune responses to human immunodeficiency virus type 1 by a DNA vaccine formulated with QS-21 saponin adjuvant via intramuscular and intranasal routes. *J. Virol.* 72: 4931–4939.
30. Sasaki, Y., Y. Ami, T. Nakasone, K. Shinohara, E. Takahashi, S. Ando, K. Someya, Y. Suzaki, and M. Honda. 2002. Induction of CD95 ligand expression on T lymphocytes and B lymphocytes and its contribution to apoptosis of CD95-upregulated $CD4^+$ T lymphocytes in macaques by infection with a pathogenic simian/human immunodeficiency virus. *Clin. Exp. Immunol.* 122: 381–389.
31. Shinohara, K., K. Sakai, S. Ando, Y. Ami, N. Yoshino, E. Takahashi, K. Someya, Y. Suzaki, T. Nakasone, Y. Sasaki, et al. 1999. A highly pathogenic simian/human immunodeficiency virus with genetic changes in cynomolgus monkeys. *J. Gen. Virol.* 80: 1231–1240.
32. Yoshino, N., T. Ryu, M. Sugamata, T. Ihara, Y. Ami, K. Shinohara, F. Tashiro, and M. Honda. 2000. Direct detection of apoptotic cells in peripheral blood from highly pathogenic SHIV-inoculated monkeys. *Biochem. Biophys. Res. Commun.* 268: 868–874.
33. Lu, Y., M. S. Salvato, C. D. Pauza, J. Li, J. Sodroski, K. Manson, M. Wyand, N. Letvin, S. Jenkins, N. Touzjian, et al. 1996. Utility of SHIV for testing HIV-1 vaccine candidates in macaques. *J. Acquired Immune Defic. Syndr. Hum. Retrovirol.* 12: 99–106.
34. Reimann, K. A., J. T. Li, G. Voss, C. Lekutis, K. Tenner-Racz, P. Racz, W. Lin, D. C. Montefiori, D. E. Lee-Parritz, Y. Lu, et al. 1996. An *env* gene derived from a primary human immunodeficiency virus type 1 isolate confers high in vivo replicative capacity to a chimeric simian/human immunodeficiency virus in rhesus monkeys. *J. Virol.* 70: 3198–3206.
35. Mothe, B. R., H. Horton, D. K. Carter, T. M. Allen, M. E. Liebl, P. Skinner, T. U. Vogel, S. Fuenger, K. Vielhunber, W. Rehrauer, et al. 2002. Dominance of CD8 responses specific for epitopes bound by a single major histocompatibility complex class I molecule during the acute phase of viral infection. *J. Virol.* 76: 875–884.
36. Barouch, D. H., S. Santra, J. E. Schmitz, M. J. Kuroda, T. M. Fu, W. Wagner, M. Bilska, A. Craiu, X. X. Zheng, G. R. Krivulka, et al. 2000. Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. *Science* 290: 486–492.

37. Carroll, M. W., and B. Moss. 1997. Host range and cytopathogenicity of the highly attenuated MVA strain of vaccinia virus: propagation and generation of recombinant viruses in a nonhuman mammalian cell line. *Virology* 238: 198–211.
38. Ober, B. T., O. Bruhl, M. Schmidt, V. Wieser, W. Gritschenberger, S. Coulibaly, H. Savidis-Dacho, M. Gerencer, and F. G. Falkner. 2002. Immunogenicity and safety of defective vaccinia virus lister: comparison with modified vaccinia virus Ankara. *J. Virol.* 76: 7713–7723.
39. Ami, Y., Y. Izumi, K. Matsuo, K. Someya, M. Kanekiyo, S. Horibata, N. Yoshino, K. Sakai, K. Shinohara, S. Yamazaki, et al. 2005. Prime-boost vaccination with recombinant *Mycobacterium bovis* bacillus Calmette Guérin and a non-replicating vaccinia virus recombinant leads to long-lasting and effective immunity. *J. Virol.* 79: 12871–12879.
40. Putkonen, P., M. Quesada-Rolander, A. C. Leandersson, S. Schwartz, R. Thorstensson, K. Okuda, B. Wahren, and L. Hinkula. 1998. Immune responses but no protection against SHIV by gene-gun delivery of HIV-1 DNA followed by recombinant subunit protein boosts. *Virology* 250: 293–301.
41. Vogel, T. U., M. R. Reynolds, D. H. Fuller, K. Vielhuber, T. Shipley, J. T. Fuller, K. J. Kunstman, G. Sutter, M. L. Marthas, V. Erfle, et al. 2003. Multispecific vaccine-induced mucosal cytotoxic T lymphocytes reduce acute-phase viral replication but fail in long-term control of simian immunodeficiency virus SIVmac239. *J. Virol.* 77: 13348–13360.
42. Baker, E. 1997. $CD8^+$ cell-derived anti-human immunodeficiency virus inhibitory factor. *J. Infect. Dis.* 179 (Suppl. 3): S485–S488.
43. Zhang, L., W. Yu, T. He, J. Yu, R. E. Caffrey, E. A. Dalmasso, S. Fu, T. Pham, J. Mei, J. J. Ho, et al. 2002. Contribution of human α-defensin 1, 2, and 3 to the anti-HIV-1 activity of CD8 antiviral factor. *Science* 298: 995–1000.
44. Musey, L. K., J. N. Krieger, J. P. Hughes, T. W. Schacker, L. Corey, and M. J. McElrath. 1999. Early and persistent human immunodeficiency virus type 1 (HIV-1)-specific T helper dysfunction in blood and lymph nodes following acute HIV-1 infection. *J. Infect. Dis.* 180: 278–284.
45. Rosenberg, E. S., J. M. Billingsley, A. M. Caliendo, S. L. Boswell, P. E. Sax, S. E. Kalams, and B. D. Walker. 1997. Vigorous HIV-1-specific $CD4^+$ T cell responses associated with control of viremia. *Science* 278: 1447–1450.
46. Wilson, J. D., N. Imami, A. Watkins, J. Gill, P. Hay, B. Gazzard, M. Westby, and F. M. Gotch. 2000. Loss of $CD4^+$ T cell proliferative ability but not loss of human immunodeficiency virus type 1 specific equates with progression to disease. *J. Infect. Dis.* 182: 792–798.
47. Matloubian, M., R. J. Conception, and R. Ahmed. 1994. $CD4^+$ T cells are required to sustain $CD8^+$ cytotoxic T-cell responses during chronic viral infection. *J. Virol.* 68: 8056–8063.
48. Walter, E. A., P. D. Greenberg, M. J. Gilbert, R. J. Finch, K. S. Watanabe, E. D. Thomas, and S. R. Riddell. 1995. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clone from the donor. *N. Engl. J. Med.* 338: 1038–1044.
49. Estcourt, M. J., A. J. Ramsay, A. Brooks, S. A. Thomson, C. J. Medveckz, and I. A. Ramshaw. 2002. Prime-boost immunization generates a high frequency, high-avidity $CD8^+$ cytotoxic T lymphocyte population. *Int. Immunol.* 14: 31–37.
50. Edwards, B. H., A. Bansal, S. Sabbaj, J. Bakari, M. J. Mulligan, and P. A. Goepfert. 2002. Magnitude of functional $CD8^+$ T-cell responses to the gag protein of human immunodeficiency virus type 1 correlates inversely with viral load in plasma. *J. Virol.* 76: 2298–2305.
51. Hel, Z., J. Nacsa, E. Tryniszewska, W. P. Tsai, R. W. Parks, D. C. Montefiori, B. K. Felber, J. Tartaglia, G. N. Pavlakis, and G. Franchini. 2002. Containment of simian immunodeficiency virus infection in vaccinated macaques: correlation with the magnitude of virus-specific pre- and after challenge $CD4^+$ and $CD8^+$ T cell responses. *J. Immunol.* 169: 4478–4487.
52. Seth, A., I. Ourmanov, M. J. Kuroda, J. E. Schmitz, M. W. Caroll, L. S. Wyatt, B. Moss, M. A. Forman, V. M. Hirsch, and N. L. Letvin. 1998. Recombinant modified vaccinia virus Ankara-simian immunodeficiency virus gag pol elicits cytotoxic T lymphocytes in rhesus monkeys by a major histocompatibility complex class I/peptide tetramer. *Proc. Natl. Acad. Sci. USA* 95: 10112–10116.
53. Robinson, S., W. A. Charini, M. H. Newberg, M. J. Kuroda, C. I. Lord, and N. L. Letvin. 2001. A commonly recognized simian immunodeficiency virus Nef epitope presented to cytotoxic T lymphocytes of Indian-origin rhesus monkeys by the prevalent major histocompatibility complex class I allele Mamu-A*02. *J. Virol.* 75: 10179–10186.
54. Seaman, M. S., S. Santra, M. H. Newberg, V. Philippon, K. Manson, L. Xu, R. S. Gelman, D. Panicali, J. R. Mascola, G. J. Nabel, et al. 2005. Vaccine-elicited memory cytotoxic T lymphocytes contribute to Mamu-A*01-associated control of simian/human immunodeficiency virus 89.6P replication in rhesus monkeys. *J. Virol.* 79: 4580–4588.
55. Miller, M. D., H. Yamamoto, A. L. Hughes, D. I. Watkins, and N. L. Letvin. 1991. Definition of an epitope and MHC class I molecule recognized by gag-specific cytotoxic T lymphocytes in SIV mac-infected rhesus monkeys. *J. Immunol.* 147: 320–329.
56. Yamamoto, H., M. D. Miller, H. Tubota, D. I. Watkins, G. P. Mazzara, V. Stallard, D. L. Panicali, A. Aldovini, R. A. Young, and N. L. Letvin. 1990. Studies of cloned simian immunodeficiency virus-specific T lymphocytes: gag-specific cytotoxic T lymphocytes exhibit a restricted epitope specificity. *J. Immunol.* 144: 3385–3389.
57. Morita, M., Y. Aoyama, M. Arita, H. Amona, H. Yoshizawa, S. Hashizume, T. Komatsu, and I. Tagaya. 1977. Comparative studies of several vaccinia virus strains by intrathalamic inoculation into cynomolgus monkeys. *Arch. Virol.* 53: 197–208.
58. Allen, T. M., P. Jing, P., B. Calore, H. Horton, D. H. O'Connor, T. Hanke, M. Piekarczyk, R. Ruddersdorf, B. R. Mothe, C. Emerson, et al. 2002. Effects of cytotoxic T lymphocytes (CTL) directed against a single simian immunodeficiency virus (SIV) Gag CTL epitope on the course of SIVmac239 infection. *J. Virol.* 76: 10507–10511.
59. Horton, H., T. U. Vogel, D. K. Carter, K. Vielhuber, D. H. Fuller, T. Shipley, J. T. Fuller, K. J. Kunstman, G. Sutter, D. C. Montefiori, et al. 2002. Effects of cytotoxic T lymphocytes (CTL) directed against a single simian immunodeficiency virus (SIV) Gag CTL epitope on the course of SIVmac239 infection. *J. Virol.* 76: 7187–7202.
60. Gruters, R. A., F. G. Terpstra, R. E. De Goede, J. W. Mulder, F. De Wolf, P. T. Schellekens, R. A. Van Lier, M. Tersmette, and F. Miedema. 1991. Immunological and virological markers in individuals progressing from seroconversion to AIDS. *AIDS* 5: 837–844.
61. Gruters, R. A., F. G. Terpstra, R. De Jong, C. J. Van Noesel, R. A. Van Lier, and F. Miedema. 1990. Selective loss of T cell function in different stages of HIV infection: early loss of anti-CD3-induced T cell proliferation followed by decreased anti-CD3-induced cytotoxic T lymphocyte generation in AIDS-related complex and AIDS. *Eur. J. Immunol.* 20: 1039–1044.
62. Jaleco, A. C., M. J. Covas, L. A. Pinto, and R. M. Victorino. 1994. Distinct alterations in the distribution of $CD45RO^+$ T-cell subsets in HIV-2 compared with HIV-1 infection. *AIDS* 8: 1663–1666.
63. Schnittman, S. M., H. C. Lane, J. Greenhouse, J. S. Justement, M. Baseler, and A. S. Fauci. 1990. Preferential infection of $CD4^+$ memory T cells by human immunodeficiency virus type 1: evidence for a role in the selective T-cell functional defects in infected individuals. *Proc. Natl. Acad. Sci. USA* 87: 6058–6062.
64. Van Noesel, C. J., R. A. Gruters, F. G. Terpstra, P. T. Schellekens, R. A. Van Lier, and F. Miedema. 1990. Functional and phenotypic evidence for a selective loss of memory T cells in asymptomatic human immunodeficiency virus-infected men. *J. Clin. Invest.* 86: 293–299.
65. Ginaldi, L., M. De Martinis, A. D'Ostilio, A. Di Gennaro, L. Marini, V. Profeta, and D. Quaglino. 1997. Activated naive and memory $CD4^+$ and $CD8^+$ subsets in different stages of HIV infection. *Pathobiology* 65: 91–99.
66. Miedema, F. 1992. Immunological abnormalities in the history of HIV infection: mechanisms and clinical relevance. *Immunodefic. Rev.* 3: 173–193.
67. Ullum, H., A. C. Lepri, J. Victor, P. Skinhoj, A. N. Phillips, and B. K. Pedersen. 1997. Increased losses of $CD4^+CD45RA^+$ cells in late stages of HIV infection is related to increased risk of death: evidence from a cohort of 347 HIV-infected individuals. *AIDS* 11: 1479–1485.
68. Choi, B. S., Y. K. Park, and J. S. Lee. 2002. The CD28/HLA-DR expression on $CD4^+$ T but not $CD8^+$ T cells are significant predictor for progression to AIDS. *Clin. Exp. Immunol.* 127: 137–144.
69. Kammerer, R., A. Iten, P. C. Feri, and P. Burgisser. 1996. Expansion of T cells negative for CD28 expression in HIV infection: relation to activation markers and cell adhesion molecules, and correlation with prognostic markers. *Med. Microbiol. Immunol.* 185: 19–25.

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# Intradermal and oral immunization with recombinant *Mycobacterium bovis* BCG expressing the simian immunodeficiency virus Gag protein induces long-lasting, antigen-specific immune responses in guinea pigs

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Human dose;
Guinea pig

**Abstract** To develop a new recombinant BCG (rBCG) vaccine, we constructed rBCG that expresses the full-length Gag protein of simian immunodeficiency virus (rBCG-SIVGag) at a level of 0.5 ng/mg after 3 weeks of bacterial cell culture. Intradermal (i.d.) inoculation of guinea pigs with 0.1 mg of rBCG-SIVGag resulted in the induction of delayed-type hypersensitivity (DTH) responses to both purified protein derivative (PPD) of tuberculin and SIV Gag p27 protein; responses that were maintained for the duration of the 50-week study. In contrast, guinea pigs orally vaccinated with 160 mg of the same antigen exhibited a long-lasting DTH response to the SIV Gag p27 protein, but mounted no response to PPD. Proliferative responses to SIV Gag p27 and PPD antigens were detected in both i.d. and orally immunized animals; however, the levels of PPD-specific responses were significantly higher in guinea pigs immunized by the i.d. than the oral route. A significant increase in the level of PPD- and SIV Gag p27-specific IFNγ mRNA expression was also detected in both immunization groups receiving rBCG-SIVGag. In addition, both i.d. and oral immunization with rBCG-SIVGag induced PPD- and SIV Gag p27-specific serum IgG responses. Insertion of the SIV *gag* gene into BCG did not appear to change the ability of rBCG-immunized animals to elicit PPD-specific immune responses. These results indicate that rBCG-SIVGag has the ability to effectively induce long-lasting, cell-mediated and humoral immunity against both viral and bacterial antigens in guinea pigs, suggesting that rBCG-Gag has the potential to elicit immunities specific not only for tuberculosis but also for HIV at human doses.
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## Introduction

The epidemic of human immunodeficiency virus type 1 (HIV-1) infection and AIDS (HIV/AIDS) continues to spread worldwide, particularly in Asia and Africa. Globally, 40 million people are now living with HIV/AIDS [1]. In 2003, there were five million new HIV-1 infections, over 90% of them in developing countries [1] where rapid disease progression is more likely to occur due to co-infection with both HIV-1 and *Mycobacterium tuberculosis*. The best hope for individuals living in these countries is the development of a safe, effective and affordable vaccine to prevent HIV-1 infection. Despite recent advances in medical treatments for HIV-1, including highly active anti-retroviral therapy (HAART), most therapeutic drugs remain prohibitively expensive and inaccessible to people living in countries hardest hit by the epidemic [1].

With this dilemma in mind, our group has developed a recombinant BCG (rBCG)-vector system designed to address both the problem of HIV-1 and *M. tuberculosis* co-infection and the issue of cost facing those in developing countries. The rBCG-vector system has been shown to induce immune responses against both HIV-1 and *M. tuberculosis*, and its use is supported by a number of studies demonstrating efficacy in the induction of antigen-specific immunity. For example, it has been reported that BCG and its cell wall components possess adjuvant properties for enhancing the immunogenicity of an antigen when administered to animals [2 4]. Moreover, rBCG expressing HIV-1 antigens can act simultaneously as both an adjuvant and a vehicle to induce antigen-specific immunity [5]. Our own group has previously demonstrated that rBCG containing a 19-amino-acid insert from the HIV-1 Env V3 region (rBCG Env V3) expressed sufficient V3 antigen to induce HIV-1-specific cell-mediated and humoral immune responses in a small-animal model [6 9]. In addition, several groups have also shown the induction of cellular and/or humoral immune responses by inoculation with rBCG expressing HIV or simian immunodeficiency virus (SIV) proteins [10 14]. However, 10- to 100-fold higher doses than that needed for a common BCG vaccination against tuberculosis in humans, or repeated inoculations, were needed to effectively elicit HIV- or SIV-specific immunity in animal models [6 14]. Moreover, previous studies often used intravenous or subcutaneous routes of inoculation; however, vaccination regimens such as these are not practical for use in humans in terms of safety. Furthermore, these BCG recombinants contained a single epitope from HIV or SIV; however, it was reported that rBCG expressing a SIV gag single epitope failed to protect macaques against intravenous challenge with SIV [15].

One of the strategies to practically use a rBCG-based HIV vaccine is to inoculate 0.1 mg of the vaccine into humans via intradermal (i.d.) route as a priming or boosting immunogen because the dose and route of immunization is commonly used for BCG vaccination in humans. For this purpose, we sought to construct a novel rBCG capable of effectively inducing long-lasting, virus-specific immunity by a single i.d. vaccination with 0.1 mg. To elicit antigen-specific immunity with a multi-epitope rBCG vaccine, we chose to target HIV-1 Gag based on evidence of several cytotoxic T lymphocyte (CTL) epitopes in this region [16], some of which are MHC-linked and known to be immunodominant and relatively conserved among various HIV-1 clades [16 19]. Recently, it was shown that Gag-specific T helper cells and CTL correlate inversely with the level of plasma HIV-1 RNA [20 22]. These findings suggest that the HIV-1 Gag region is strongly immunogenic and may induce effective anti-viral responses.

In the present study, we inserted the full-length *gag* gene of SIV into BCG to create rBCG-SIVGag. We then investigated its ability to elicit antigen-specific immune responses in guinea pigs immunized either intradermally (i.d.) or orally with rBCG-SIVGag at human doses and assessed the possibility of the replacement of common BCG vaccination (0.1 mg by i.d. inoculation) by administration of a rBCG-based vaccine.

## Subjects and methods

### Animals

Female guinea pigs of the Hartley strain (Shizuoka Laboratory Center, Shizuoka, Japan), weighing 200 to 250 g each, were used in a P2-level animal facility at the National Institute of Infectious Diseases (NIID), Tokyo, Japan. The animals were fed in a specific pathogen-free level 2 facility according to NIID animal care guidelines. The study was conducted in the experimental animal area of a biosafety level 2 NIID facility under the guidance of an institutional committee for biosafety and animal experiments.

### Construction of a plasmid containing the full-length SIV *gag* gene

A recombinant *Mycobacterium bovis* BCG substrain Tokyo was produced by transfection of BCG-Tokyo strain cells with either the plasmid pSO246 [23] or pSO246SIVGag. The SIVmac239 *gag* gene [24] was amplified by PCR from simian immunodeficiency virus DNA [25] using primers 5′-CCCGGATCCATGGGCGTGAGAAACTCC-3′ (forward) and 5′-CCGCCCGGGCTACTGGTCTCCTCCAAAGAG-3′ (reverse). The resulting PCR product was inserted into the multi-cloning site of pSO246 under control of the *hsp60* promoter of BCG [26]. BCG was transformed with the recombinant plasmid by electroporation and selected on Middlebrook 7H10 agar (BBL Microbiology Systems, Cockeyville, MD) containing 10% OADC enrichment (BBL Microbiology Systems) and 20 μg/ml kanamycin. The resulting recombinant clones containing either pSO246SIVGag or pSO246 were designated rBCG-SIVGag and rBCG-pSO246, respectively.

### Western blot and ELISA detection of expressed SIV Gag

Expression of the SIV Gag protein by rBCG-SIVGag was determined by both Western blot and ELISA. rBCG-SIVGag was harvested from Middlebrook 7H9 broth containing ADC (BBL Microbiology Systems) 3 weeks after initiation of the culture, when the growth curve of the transformant had reached its peak. The harvested rBCG-SIVGag was sonicated completely and centrifuged, and the supernatant was heated at 95 C for 5 min in sample buffer (10% 2-mercaptoethanol, 20% glycerol, 123.9 mM Trizma base, 138.7 mM SDS, 3.0 mM bromphenol blue). SDS-polyacryl-