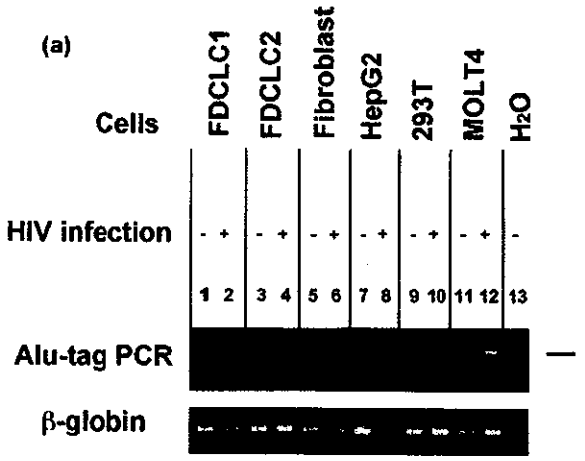

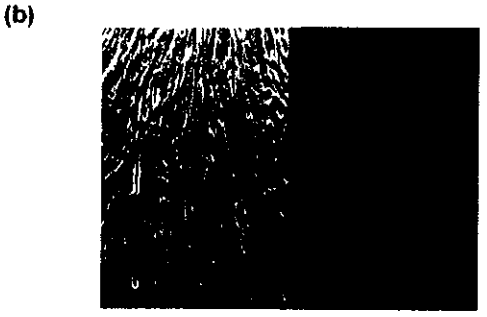

Fig. 3. FDCLCs are not the targets themselves for HIV-1 (NL4-3). (a) Alu-tag and β-globin PCR were performed with DNA extracts of FDCLC-1 (lanes 1, 2), FDCLC-2 (lanes 3, 4), fibroblast (lanes 5, 6), HepG2 (lanes 7, 8), 293T (lanes 9, 10), and MOLT-4 (lanes 11, 12). Cells were treated with NL4-3 (lanes 2, 4, 6, 8, 10, 12) or heat-inactivated NL4-3 (lanes 1, 3, 5, 7, 9, 11) for 2 hr at 37 C and washed. DNA of HIV-1-exposed cells was individually extracted 7 days after the incubation. Positive control was shown in lane 12 while negative controls were lanes 1, 3, 5, 7, 9, 11 and H_2O (lane 13). FDCLC1 and 2 showed no PCR products (lanes 2, 4) as well as fibroblast (lane 6), HepG2 (lane 8), and 293T (lane 10). (b) FDCLC were exposed to VSV pseudo-type GFP-tagged HIV-1 for 2 hr at 37 C. After incubation, FDCLC were washed by medium twice. GFP-positive FDCs indicated integration of the HIV-1.

DNA could be detected in DNA extract of HIV-1-exposed MOLT-4 used as the positive control (Fig. 3(a)). When MOLT-4 was treated with heat-inactivated NL4-3, neither integrated HIV-1 DNA nor p24-increase in the culture supernatant was observed 7 days later (data not shown). On the other hand, almost all FDCLC were infected with VSV-env single round

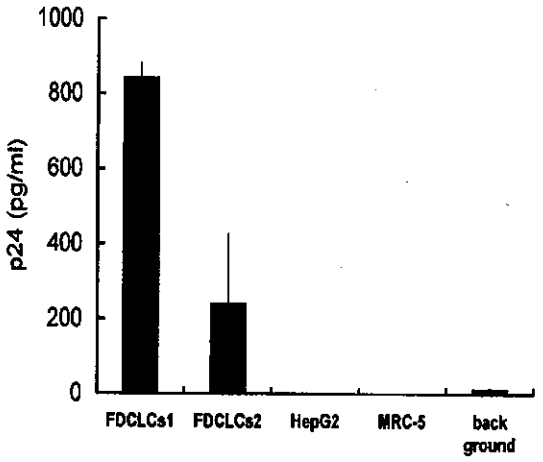

Fig. 4. HIV-1 pre-exposed FDCLC could transmit virus to MOLT-4 cells. FDCLC, HepG2 and MRC-5 cells were treated with NL4-3 for 2 hr at 37 C and washed. One day after, they were co-cultured with MOLT-4 T cells. P24 levels in the supernatants were measured by Lumipulse 6 days after the co-culture. The data represent means± the standard deviations for triplicate wells in each condition.

pseudotyped virus and replicated virus (Fig. 3 (b)). These data indicated that FDCLC were not the target for efficient HIV-1 replication due to the failure of entry.

To examine whether FDCLC could trap and transmit HIV-1 to T cells and how long they could maintain infectious HIV-1, p24 levels were measured in culture-supernatants of FDCLC pre-exposed to HIV-1 and then co-cultured with MOLT-4. Figure 4 shows a significant increase in p24 levels 6 days after setting when FDCLC were pre-exposed to HIV-1 and 1 day after co-cultured with MOLT-4. MRC-5 and HepG2 cells did not show HIV-1 amplification. Similarly, the FDCLC exposed to virus but not co-cultured with MOLT-4 showed no increase in p24 level even after 6 days. The supportive effect of the FDCLC for viral replication of co-cultured MOLT-4 T cells worked during only 2 days after exposure to NL4-3. In microscopy, MOLT-4 often clustered on FDCLC. Co-cultures of HIV-1-exposed FDCLC with MOLT-4 separated from the former through a pored membrane gave no p24-increase (data not shown). These data suggest FDC successfully trap and transmit virus to MOLT-4 via cell-to-cell contact.

FDCLCs Enhance Viral Replication in T Cells Infected with HIV-1

When MOLT-4 were pre-infected with HIV-1 and then co-cultured with FDCLC, virus production was significantly accelerated as compared with co-cultures with MRC-5 and HepG2 or MOLT-4 only. This

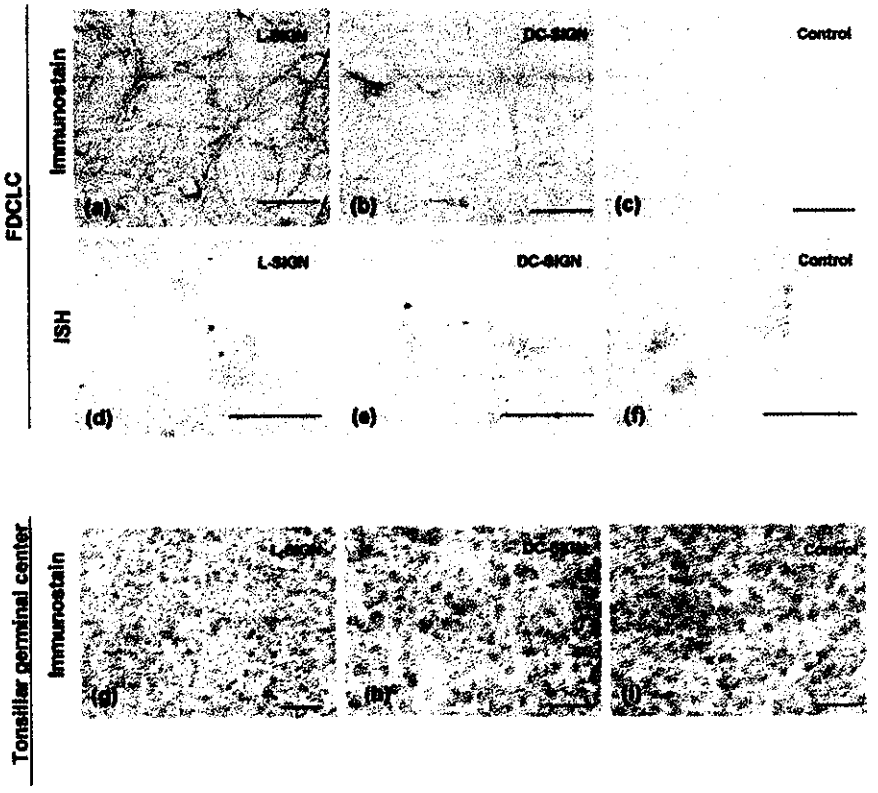

Fig. 2. Expression of L-SIGN and DC-SIGN in FDCLC and FDC in tonsillar GC. Immunostaining studies were carried out for L-SIGN (a), DC-SIGN (b) and control (c). *In situ* hybridizations for L-SIGN (d), DC-SIGN (e) and control (f). Frozen sections of tonsil GC for L-SIGN (g), DC-SIGN (h) and control (i). Each bar indicates 100 μm.

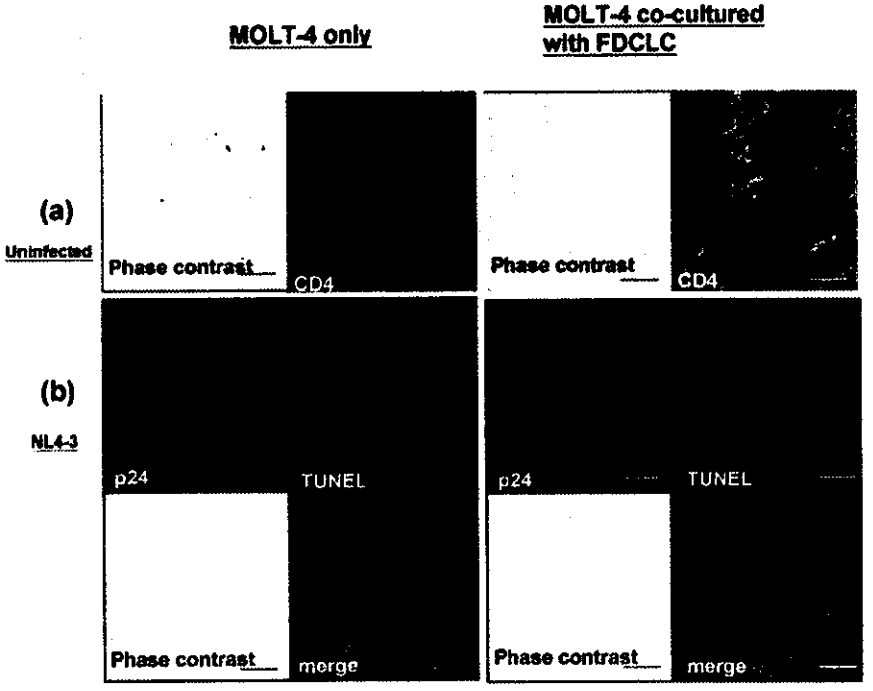

Fig. 6.

increase in virus production was time dependent (Fig. 5). The P24 level was 6,683 pg/ml 10 days after co-culture, while those done with HepG2 or MRC-5 were 777 or 1,788 pg/ml, respectively (Fig. 5). Infected MOLT-4 cultured alone produced 993 pg/ml under the present experimental condition. Similar result was obtained with another strain of FDCLC (FDCLC-2). The co-culture through a membrane showed no enhancement of p24-production (data not shown). Cytokine stimulations with IL-1β and TNF-α gave no additional effect on this phenomenon (data not shown). Our results indicate FDCLC enhanced HIV-1 replication and p24 production by infected MOLT-4 cells when co-cultured.

FDCLCs Up-Regulate CD4 Expression in MOLT-4 in the Co-Cultures and Inhibited T Cell from Apoptosis

To ascertain the mechanism of enhanced viral replication in T cells, we analyzed co-cultured FDC and HIV-1-non-infected or -infected MOLT-4 by immunofluorescent confocal microscopy. In the absence of HIV-1, FDCLC rather up-regulated expression of CD4, the primary receptor for HIV-1, in MOLT-4 cells 5 days after co-culture (Fig. 6 (a)). When MOLT-4 was infected with NL4-3, CD4 expression in MOLT-4 apparently down-regulated 5 days after culture with or without FDCLC (data not shown). Furthermore, we carried out a double staining for p24 and TUNEL to address the relationship between virus production and cell death. In comparison with HIV-1-infected MOLT-4 alone, those co-cultured with FDCLC showed a significant increase not only in frequency of the p24-positive cells but also in intensity of their individual positive dots. Interestingly, the co-culture with FDCLC resulted in a decrease in number of TUNEL-positive apoptotic MOLT-4 and of syncytial giant cells as compared with those cultured alone (Fig. 6 (b)). The merged images indicated most of apoptotic cells were p24-negative especially in the co-culture system. FDCLC seemed to accelerate HIV-1 replication in MOLT-4 through promoting survival of T cells.

Discussion

Amounts of HIV-1 accumulated in FDC-NW in GC ultimately become a source for subsequent virus replication and genomic variation, not only in the later stage with specific antibody but also shortly after HIV-1 infection (8, 15, 29). Although distortion of FDC-NW known as follicle lysis (14) is a hallmark of immunodeficiency, it remained to be elucidated how FDC engage in the early HIV-1 infection. To address this, we established an *in vitro* model of the early infection with

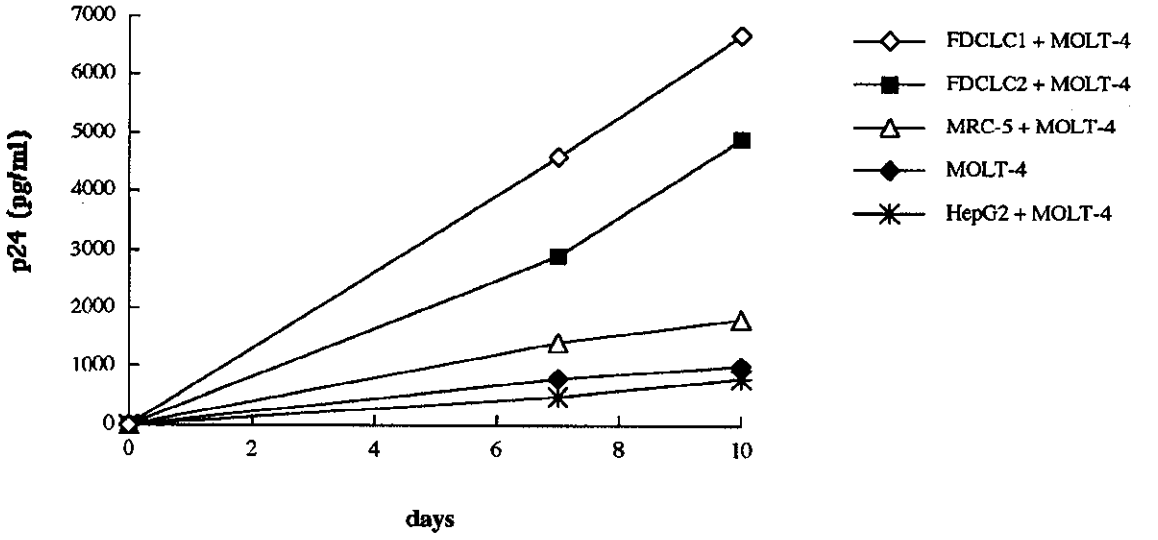

Fig. 5. FDCLCs enhance HIV-1 production from MOLT-4 cells. MOLT-4 cells were incubated with NL4-3 virus for 2 hr at 37 C, washed and cultured or co-cultured with FDCLC or MRC-5 or HepG2 cells. After 10 days, p24 levels were measured.

Fig. 6. Confocal microscopy of HIV-1-non-infected or -infected MOLT-4 and those co-cultured with FDCLC. (a) Phase contrast microscopy (left) and CD4-immunostaining (right) in HIV-1-non-infected MOLT-4 cells and those co-cultured with FDCLC. (b) Double-staining of p24 and TUNEL in infected MOLT-4 cultured alone or those co-cultured with FDCLC. HIV-1-infected MOLT-4 alone or those co-cultured with FDCLC were fixed by PLP 5 days after. Infected MOLT-4 co-cultured with FDCLC show high expression of p24 (FITC, green) and low level of TUNEL-positive cells (Alexa-red). Inversely, infected MOLT-4 alone show high frequency of TUNEL-positive apoptotic cells with less intense p24 signals.

FDCLC and MOLT-4. Unique features of FDCLC are summarized as follows: 1) inability of FDCLC to integrate and replicate HIV-1, 2) ability to trap HIV-1 and transmit HIV-1 to T cells and 3) ability to enhance HIV-1 production in pre-infected T cells through escape of T cells from apoptosis. FDCLC showed a phenotype positive for CNA-42, S-100α and a fibroblastic antigen similar to the cells previously reported and very resembled those reported by Choi's group (10). Based on these results, they are estimated to be an immature form of FDC (26). Although some markers specific for FDC were down-expressed, it was inevitable in the *in vitro*-cultured FDC. Desmosome-like or gap junctions and abundance in filiform-cytoplasmic extensions or loosened labyrinth-like structures (9) further support FDCLC are of FDC origin. A negative finding of FDCLC for CD11c and S-100β indicates that they were not DC (7). A sub-strain of FDCLC selected with MoAb DRC-1-coated beads showed the same findings in immunohistochemical and virological tests as those of FDCLC (data not shown). As additional data to prove our FDCLC were of FDC origin, we observed that fluorescence (PKH-26)-labeled FDCLC given i.v. in adult C57BL/NJ and hSCID (NOG) mice localized in their GC or lymphoid follicle of the spleen respectively. The FDCLC interestingly re-expressed DRC-1, once lost immediately after the primary culture, in such transplanted mice (Terashima, unpublished).

HIV integration in FDC is controversial (6, 17, 21, 25). The novel nested 'Alu-tag' PCR to detect Alu elements-conserved genomic form of HIV-1 DNA showed no integration of the NL4-3 in our FDCLC. However, GFP-tagged NL4-3 modified with VSV-derived *env* could be integrated. We therefore recognized inability of FDC to replicate HIV-1 as previously reported *in vitro* (2, 21, 22, 25), and distinctly disclosed it was due to the inability of the virus to enter into the cytoplasm.

Besides trapping HIV-1 to transmit to T cells, FDCLC significantly promoted viral replication in infected T cells co-cultured. As MOLT-4 co-cultured with FDCLC did not exceed those cultured alone in number on day 4 and 7 after the culture (data not shown), the p24 increase in the co-culture is not due to an increase of T cells but due to the effect of FDCLC to T cells.

As a candidate of molecules engaging in such a high affinity of FDCLC/FDC to HIV-1 in the absence of immune complex and antibody, we noticed they expressed L-SIGN and DC-SIGN (CD209L and 209). DC-SIGN was reported to capture and transmit HIV-1 in DC while the L-SIGN is a similar molecule rather confining HIV to lymph sinus and liver sinusoidal endothelia (1). However, their expression was neither intense nor constitutional as found in DCs varying in intensity according to each follicle. FDCLC replated more than one week before did not show L-SIGN and DC-SIGN expression. Therefore, it remains to be disclosed if the molecules are indeed responsible for binding of HIV-1 in FDCLCs.

Confocal microscopy demonstrated that FDC provided with an environment to T cells efficiently to replicate the virus in GC irrespective of whether the T cells were initially infected with HIV-1 in GC or previously done outside GC. We demonstrated up-regulation of CD4 in T cells by FDCLC (*Fig. 6*) while CXCR4 was already reported to be up-regulated in a similar manner to $CD4^+$ $CD56^+$ $CD45RO^+$ T cells in GC (5). Such up-regulation is also reported in B cells versus FDC (4). In HIV-1 infection, MOLT-4 co-cultured with FDCLC was rather resistant to apoptosis when compared with those cultured alone. The direct cell-to-cell contact is indispensable for FDCLC efficiently to amplify viral production by the T cells because of no acceleration of viral production when co-cultured with FDCLC through a membrane (data not shown). Finally, we noted an increase of PCNA-positive HIV-1-infected T cells when co-cultured with FDCLC. They were largely in G1 to S phase. G1b phase is reported to be suitable for the completion of the HIV-1 reverse transcription process in T lymphocytes (11). These effects by FDCLC seem to be of significance since GCs were a site where HIV-1-infected CD4 T cells proliferated during cell-cycling as demonstrated by expression of PCNA or Ki-67 (22). Most CD4 T cells in GC were previously reported to be $CD57^+$ or $HNK\text{-}1^+$ (5, 28) and to be infected by HIV-1 (22).

In conclusion, the vigorous viral replication in GC in the early step of HIV-1 infection was reproduced in *in vitro* model of FDC. Anti-HIV-1 agents, including RNAi or DNA vaccines, administered *i.v.* in a form of immune-complex will reach the FDC-NW (23, 24) effectively to prevent the viral replication in GC. This model helps us explore intrinsic strategies against early HIV-1 infection through intervention of HIV-1 replication in GC.

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A Consecutive Priming-Boosting Vaccination of Mice with Simian Immunodeficiency Virus (SIV) *gag/pol* DNA and Recombinant Vaccinia Virus Strain DIs Elicits Effective Anti-SIV Immunity

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To evaluate immunity induced by a novel DNA prime-boost regimen, we constructed a DNA plasmid encoding the *gag* and *pol* genes from simian immunodeficiency virus (SIV) (SIV*gag/pol* DNA), in addition to a replication-deficient vaccinia virus strain DIs recombinant expressing SIV *gag* and *pol* genes (rDIsSIV*gag/pol*). In mice, priming with SIV*gag/pol* DNA, followed by rDIsSIV*gag/pol* induced an SIV-specific lymphoproliferative response that was mediated by a $CD4^+$-T-lymphocyte subset. Immunization with either vaccine alone was insufficient to induce high levels of proliferation or Th1 responses in the animals. The prime-boost regimen also induced SIV Gag-specific cellular responses based on gamma interferon secretion, as well as cytotoxic-T-lymphocyte responses. Thus, the regimen of DNA priming and recombinant DIs boosting induced Th1-type cell-mediated immunity, which was associated with resistance to viral challenge with wild-type vaccinia virus expressing SIV*gag/pol*, suggesting that this new regimen may hold promise as a safe and effective vaccine against human immunodeficiency virus type 1.

As human immunodeficiency virus type 1 (HIV-1) continues to spread throughout the world (16, 27, 51, 42), the need for a safe and effective prophylactic vaccine is more urgent now than ever (16, 27, 43, 51). A realistic goal for such a vaccine is to limit HIV-1 infection by eliciting immune responses that reduce the viral load and prevent disease progression. With other viral diseases, T-helper-cell type 1 (Th1)-mediated immune responses and $CD8^+$ cytotoxic T lymphocytes (CTL) have been reported to provide protection and reduce disease progression (11, 17, 23, 34, 45, 55, 77). Moreover, Th1/$CD8^+$ T-cell responses have been shown to play an important role in controlling HIV-1 replication (9, 28, 41, 44, 50, 52, 56, 60, 61, 62). In previous studies, nonhuman primates and chimpanzees immunized with attenuated strains of simian immunodeficiency virus (SIV) or HIV-1 had strong antigen-specific immune responses and were protected from challenge with SIV, simian/human immunodeficiency virus (SHIV), or HIV-1 (1, 15, 66, 81). These studies demonstrate that an experimental immunogen is capable of mediating protection against intravenous and mucosal viral challenge in animal models of HIV and SIV, although attenuated HIV-1 vaccines are generally considered to be unsafe for use in humans (4, 21).

Recently, HIV-1 DNA-based vaccines have been shown to induce protective T-cell-mediated immune responses (12, 13, 33, 57, 75). To increase vaccine efficacy, DNA has also been modified by codon optimization, as well as by coinjection with cytokine-encoding plasmids, recombinant proteins, and other vaccine vectors (7, 10, 29, 32, 46). The immune response to DNA vaccines based on HIV-1 antigen genes was increased when innate and adaptive cytokine genes were combined (5, 14, 22). Furthermore, enhanced levels of protection were demonstrated with a combination regimen consisting of DNA priming (SIV *gag*, *pol*, *vif*, *vpx*, and *vpr*, and HIV-1 *env*, *tat*, and *rev*), followed by boosting with a recombinant modified vaccinia virus Ankara (MVA)-based vaccine (SIV*gag* and *pol* and HIV-1 *env*) (2).

Recombinant MVA has been used frequently as a booster vaccine in various combination regimens. In an effort to develop additional safe booster antigens, we generated a recombinant vaccine based on the vaccinia virus strain DIs, which has proven not to replicate in all mammalian cells tested (25). The virologic and immunologic properties of the DIs vector have been reported previously by our group (25, 26, 38, 70, 71). The vaccinia virus DIs vector expressing SIV Gag protein elicited immune responses able to suppress SHIV infection in macaques (26). In the present study, we have demonstrated enhanced Th1-type immune induction in mice primed with a plasmid DNA vaccine encoding SIV*gag/pol*, followed by boosting with a newly developed recombinant DIs strain that expresses SIV*gag/pol* (rDIsSIV*gag/pol*). Our result demonstrates that this new prime-boost regimen is both safe and effective at eliciting anti-immunodeficiency viral immunity, suggesting its promise as a potential vaccine against HIV-1.

MATERIALS AND METHODS

Animals. Female BALB/c mice ($H\text{-}2^d$) were obtained from Shizuoka Laboratory Center (Shizuoka, Japan) and were used at between 8 and 12 weeks of age.

Construction of SIV*gag/pol* encoding plasmid DNA. Plasmid DNA encoding SIV*gag/pol* was prepared by standard procedures as previously described (47, 48, 64). Briefly, SIV*gag/pol* DNA was derived from the eucaryotic expression vector pcDNA3.1(−) (Invitrogen, Carlsbad, Calif.). pcDNA3.1(−) was digested with XhoI and EcoRI and ligated to SIV*gag* and *pol* genes that were amplified from SHIV-C2/1 DNA (GenBank no. AF217181) with the primers 5′-AACTCGAG

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AAGATAGAGTGGGAGATGGG and AAGAATTCAGGCTATGCCACCT CTCTA-3′. *gag/pol* genes were derived from the molecular clone SIV_{mac239}. The correct insertion of SIV*gag/pol* DNA into the plasmid was confirmed by PCR.

Generation and propagation of rDIsSIV*gag/pol*. rDIsSIV*gag/pol* was constructed based on the previously described protocol (25, 26). The SIV*gag/pol* gene was first amplified from $SHIV_{NM-3rN}$ DNA (24) by using primers SGP-5 (5′-AATACCCGGGATGGGCGTGAGAAACTC) and SGP-3 (AATAGAGC TCCTATGCCACCTCTCTAG-3′) and then subcloned into the pUCvvp7.5H vector (25, 26). A HindIII fragment encoding SIV*gag/pol* and the p7.5H promoter region were inserted into the HindIII site of a pUC/DIs transfer vector. rDIsSIV*gag/pol* and a control vector expressing the gene for LacZ (rDIsLacZ) were generated by homologous recombination and propagated in chicken embryo fibroblasts (CEF). Each virion preparation was purified by sucrose density ultracentrifugation and stored at −120°C. The expression of a 55-kDa protein corresponding to SIV Gag was confirmed by Western blotting with extracts from CEF infected with rDIsSIV*gag/pol* and anti-SIV Gag-specific monoclonal antibodies (IB6 or V10) (35).

SIV antigens. Overlapping 15-mer peptides spanning SIV Gag (with 11-amino-acid overlaps) were provided from the NIH AIDS Research and Reference Reagent Program (National Institutes of Health, Rockville, Md.). Peptides spanning Gag p27 and p15 regions were divided into eight pools and used as antigens. Purified native SIV p27 Gag protein (SIV Gag) was purchased from Advanced Biotechnologies, Inc. (Rockville, Mass.).

Lymphocyte proliferative assays. Lymphocyte proliferative assays were performed as previously described (19). One week after the final vaccination, the mice in each of five groups (see Fig. 2) were sacrificed. The spleens were removed, and the tissue was disrupted by compression through a cell strainer (Becton Dickinson, Franklin Lakes, N.J.). Isolated spleen cells were pooled and resuspended at 10^6 cells/ml in RPMI 1640 medium supplemented with 10% fetal bovine serum, L-glutamine, penicillin, and streptomycin. The $CD4^+$-T-cell, $CD8^+$-T-cell, or $CD4^+/CD8^+$-T-cell fraction was then depleted by using magnetic cell sorting (MACS; Miltenyi Biotech, Bergisch Gladbach, Germany) (20, 63). Aliquots of cells (100 μl) were transferred to 96-well round-bottom plates in triplicate. Either 1 μg of native purified SIV p27 Gag protein or pooled peptides (1 μg/peptide/10^5 cells) per ml was added to each well. The cells were incubated at 37°C in 5% CO_2 for 2 days before the addition of 1 μCi of [^{3}H]thymidine. After an additional 24 h of incubation, the cells were harvested, and the uptake of ^3H was determined. The results are expressed as the stimulation index (SI), which was calculated as a ratio of the counts per minute in the presence or absence of antigen.

Analysis of antigen-specific cytokine production. To further characterize the type of immune response induced in the vaccinated mice, $CD4^+$ T cells were isolated from the total spleen cell population by using MACS as described above. The purity of the isolated $CD4^+$ T cells was determined by flow cytometric analysis to be >98% (20, 63). The purified $CD4^+$ T cells were cultured for 3 days at a density of 10^6 cells/ml in the presence of pooled peptides or SIV Gag protein at a concentration of 10 μg/ml, along with T-cell-depleted and irradiated feeder cells at a density of 10^6 cells/ml. Culture supernatants were collected, and the concentrations of gamma interferon (IFN-γ), interleukin-2 (IL-2), IL-4, IL-5, IL-6, and IL-10 were measured by using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Amersham, Arlington Heights, Ill.).

IFN-γ-specific ELISPOT assays. SIV-specific IFN-γ-producing cells were enumerated by using an enzyme-linked immunospot assay (ELISPOT) and a murine IFN-γ ELISPOT kit (Diaclone Research, Besacon, France). Aliquots (100 μl) of cell suspensions containing 10^5 spleen cells were transferred to 96-well plates that were coated with anti-mouse IFN-γ antibody. Then, 1 μg of either SIV Gag protein or pooled peptides (1 μg/peptide/10^5 cells) was added, and the plates were incubated for 24 h at 37°C in 5% CO_2. The plates were then washed three times and incubated with biotinylated anti-mouse IFN-γ antibody for 1 h at 37°C. IFN-γ-specific cells were detected by using streptavidin-alkaline phosphatase conjugate and BCIP (5-bromo-4-chloro-3-indolylphosphate) substrate (Roche, Mannheim, Germany). Wells were imaged, and spot-forming cells (SFC) were counted by using a KS ELISPOT compact system (Carl Zeiss, Oberkochen, Germany). An SFC was defined as a large black spot with a fuzzy border (40). To determine significance levels, a baseline for each peptide pool was established by using the average and standard deviation (SD) of the number of SFC for each peptide. A threshold significance value, which corresponded to this average plus two SDs, was then determined. A response was considered positive if the number of SFC exceeded the threshold significance level of the control wells with no added peptide.

SIV Gag-specific CTL assays. Lymphocytes from the vaccinated mice were evaluated for CTL activity by using ^{51}Cr release assays as previously described by Takahashi et al. (72). In brief, spleen cells removed from red blood cells were resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum, L-glutamine, 2-mercaptoethanol, penicillin, and streptomycin. The cell suspensions containing 10^7 spleen cells were restimulated with 0.1 μg/peptide/10^7 cells of pooled peptides for 5 days. IL-2 containing rat T-cell-stimulated culture supernatant (T-STIM; Collaborative Res. Bedford, Mass.) was added 3 days after cell culture. Cells from the *H-2^d* haplotype line, M12.4.5, were used as targets. M12.4.5 cells were incubated with $Na_2^{51}CrO_4$ (3.7 MBq/10^7 cells) for 90 min at 37°C in 5% CO_2 before being pulsed with peptides (either 40 or 50 μg). After 1 h, the target cells were thoroughly washed with RPMI 1640 and then dispensed into 96-well V-bottom plates (10^4 cells/well). The in vitro-stimulated effector cells were added, and the plates were incubated for 4 h at 37°C. To determine the spontaneous or maximum release of ^{51}Cr, target cells were incubated with medium alone or treated with 2% Triton X-100, respectively. The percent specific lysis was calculated by using the following formula: (experimental release − spontaneous release)/(maximum release − spontaneous release).

SIV Gag-specific humoral responses. SIV Gag-specific antibody endpoint titer was measured by ELISA as previously described (67). ELISA plates were coated with 0.3 μg of SIV Gag protein (Advanced Biotechnologies) per well. Heat-inactivated pooled mice sera were serially diluted and then added to the ELISA plates. Gag-specific antibody bound to Gag protein was captured with alkaline phosphatase-labeled goat anti-mouse immunoglobulin G (Southern Biotechnology Associates, Inc., Birmingham, Ala.).

Evaluation of vaccine-induced immunity by virus challenge. To address whether the immune responses induced by the vaccination were protective, mice were challenged with wild-type vaccinia virus recombinant expressing SIV*gag/pol* (vv9019; National Institute for Biological Standards and Control, Blanch Lane, Hertfordshire, United Kingdom). The viral challenge was performed by using the method set forth by Belyakov et al. (8) and Qiu et al. (54). At 6 days after final vaccination, four mice in each vaccinated group and four naive mice were intraperitoneally (i.p.) challenged with 10^7 PFU of vv9019. One week after the challenge with the recombinant vaccinia virus expressing SIV*gag/pol*, the mice were sacrificed, and their ovaries were removed. The ovaries were homogenized, sonicated, and assayed for the challenge virus titer by plating serial 10-fold dilutions on a plate of CEF. After 3 days of culture, the plates were stained with 0.2% of crystal violet, and plaque were counted at each dilution. The results were expressed as the fold of the reduction in vaccinia virus titer in vaccinated mice versus the titer in naive mice.

ELISPOT assay for gag, pol, or whole vaccinia virus antigen-specific immunity. The magnitude of T-cell responses against SIV Gag, Pol, and vaccinia virus antigens was also measured by ELISPOT assay based on the recombinant vaccinia virus stimulation method described previously (74, 80). Briefly, spleen cells isolated from normal mouse were infected with 10 PFU per cell of either SIV gag-expressing wild-type vaccinia virus (vvSIV gag; National Institute for Biological Standards and Control), SIV pol-expressing wild-type vaccinia virus (vvSIV pol; National Institute for Biological Standards and Control), or nonrecombinant wild-type vaccinia virus (Vaccinia WR; National Institutes of Health) for 16 h and then fixed with paraformaldehyde. The virus antigen-expressing cells were used as stimulator cells and cultured with spleen cells of vaccinated mice at a stimulator/responder ratio of 1:2, and each antigen-specific ELISPOT assay was performed by the method described above.

Statistical analysis. Data are expressed as the mean ± the SD, and data analysis was carried out by using the StatView program (SAS Institute, Cary, N.C.). A P value of <0.05 was considered significant. The comparative analysis of animal groups subjected to different vaccine regimens was performed by using the Kruskal-Wallis H-test, followed by the Student-Newman-Keul correction.

RESULTS

Construction and expression of SIV*gag/pol* DNA and rDIsSIV*gag/pol*. The *gag/pol* region of SIV_{mac239} was inserted into two selected vectors. The first, pcDNA3.1(−), a eukaryotic expression vector, was used as the backbone of the SIV*gag/pol* DNA vaccine (Fig. 1A), and the second, pUC/DIs, was used as a transfer vector to generate rDIsSIV*gag/pol* and the control vector, rDIsLacZ (Fig. 1B). PCR was used to confirm that the SIV*gag/pol* DNA had been correctly inserted into each vector and Gag-specific Western blots were used

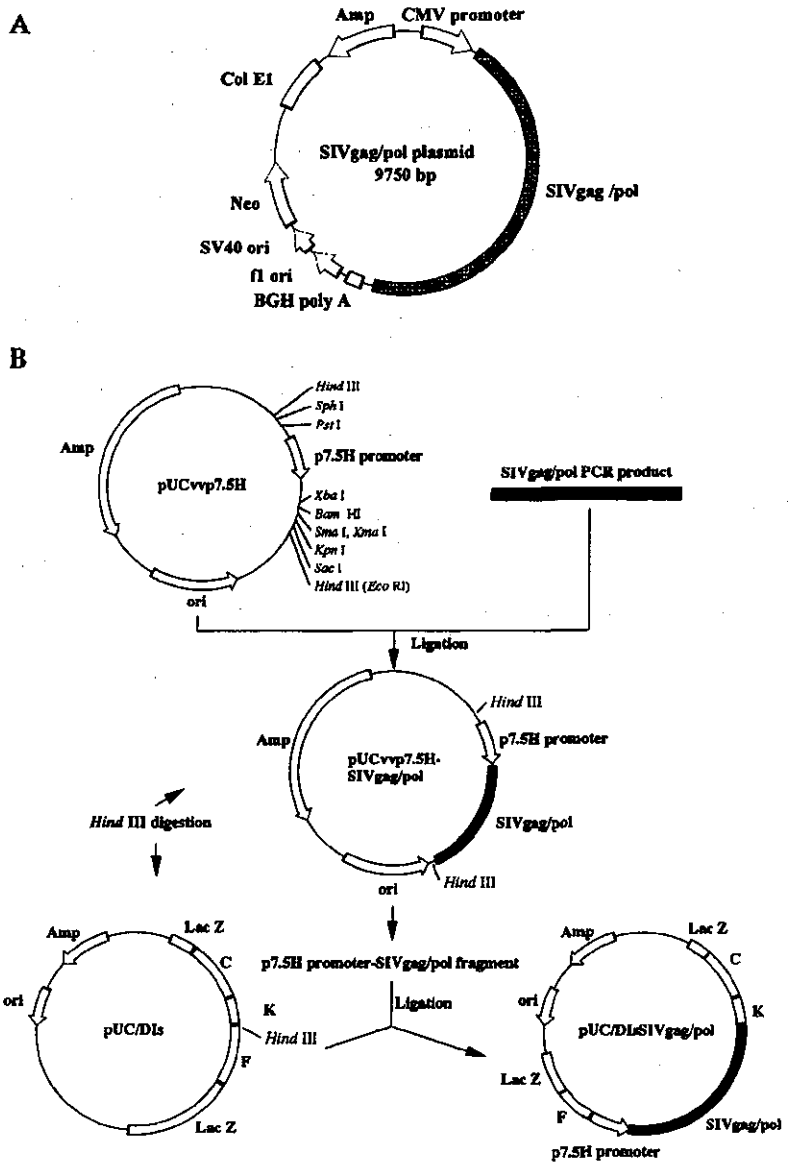

FIG. 1. Construction of SIV*gag/pol* expression vectors. (A) SIV*gag/pol* plasmid DNA; (B) construction and generation of recombinant DIsSIV*gag/pol*. The ampicillin resistance gene and vaccinia virus early/late promoter p7.5H are designated by Amp and p7.5H, respectively. Arrows indicate the direction of transcription. Hatched and white blocks represent the SIV*gag/pol* gene and HindIII fragments of vaccinia virus DNA, respectively.

to verify in vitro expression of the SIV Gag protein (data not shown).

Prime-boost regimen. A total of 50 BALB/c mice were divided into five groups of 10 mice each. Group 1 received three intramuscular injections (50 μg of each) of SIV*gag/pol* DNA at 2-week intervals, followed by two injections of rDIsLacZ (10^6 PFU each) at with 1-week intervals. Similarly, group 2 mice received three injections with 50 μg of control DNA pcDNA3.1 (−), followed by two injections of rDIsLacZ (10^6 PFU each) at 1-week intervals. Group 3 received three 50-μg intramuscular injections of SIV*gag/pol* DNA; 4 weeks later, the mice in group 3 were boosted with two intradermal injections of rDIsSIV*gag/pol* (10^6 PFU each) with a 1-week interval. The mice in group 4 received three injections of control DNA (50 μg of each), followed by two injections of rDIsSIV*gag/pol* (10^6 PFU each) (Fig. 2). In group 5, mice were immunized with SIV*gag/pol* DNA five times at the same intervals as described above. We confirmed the original data by the second run of the experiments with 50 additional animals and statistically summarized the challenge results (see Fig. 9).

The prime-boost vaccine regimen generates antigen-specific CD4$^+$-T-lymphocyte proliferative responses. SIV Gag-specific T-lymphocyte proliferative responses were measured in splenocytes from immunized mice with either SIV Gag protein or peptides spanning the full-length Gag protein of SIV_{mac239}. Spleen cells from mice in groups 1, 3, 4, and 5 showed signif-

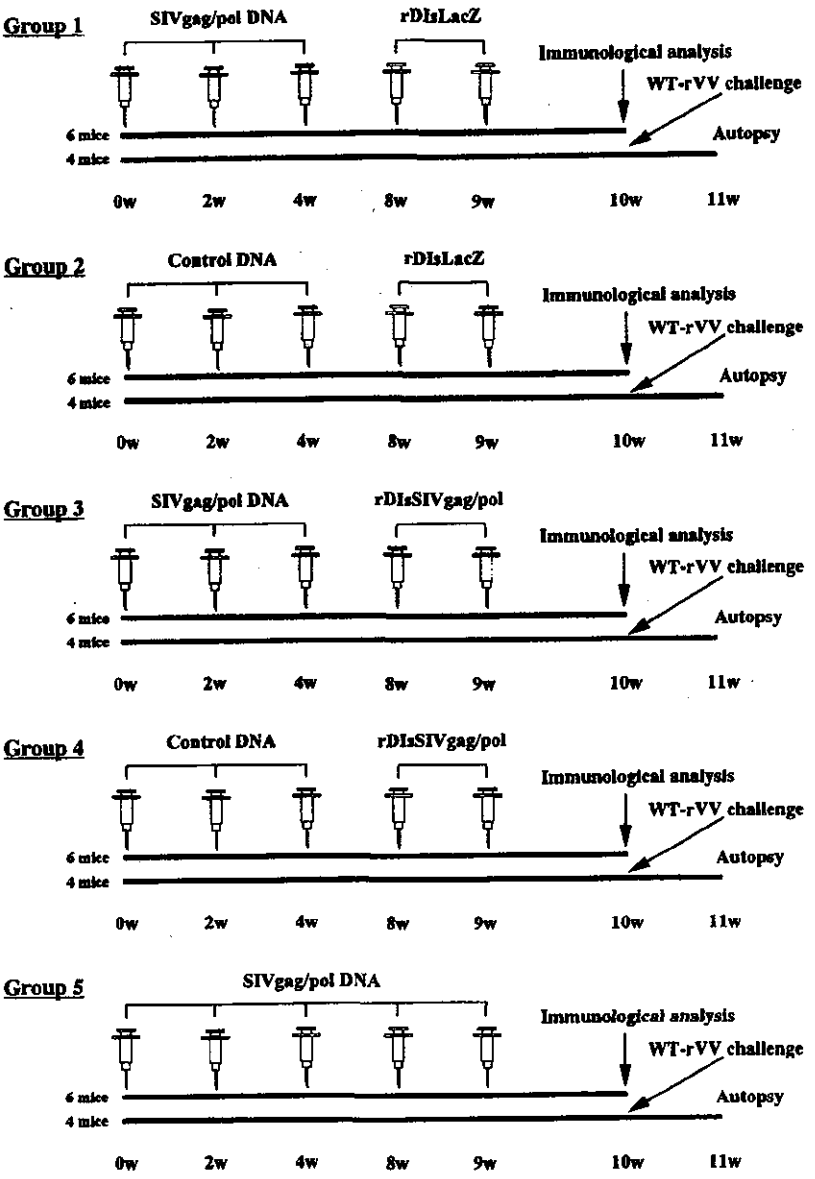

FIG. 2. Schematic of the experimental protocol for immunization and viral challenge. BALB/c mice were divided into five groups of 10 mice each (groups 1 to 5) and immunized three times consecutively with SIV*gag/pol* DNA and then twice with rDIsLacZ (group 1), three times with control DNA and then twice with rDIsLacZ (group 2), three times with SIV*gag/pol* DNA and then twice with rDIsSIV*gag/pol* (group 3), three times with control DNA and then twice with rDIsSIV*gag/pol* (group 4), or five times with SIV*gag/pol* DNA (group 5).

icant levels of proliferation in response to stimulation with SIV Gag protein and peptide pools (Fig. 3), whereas no proliferative activity (SI < 3) was seen with splenocytes from control group 2. Among the five animal groups, splenocytes from the mice in group 3 (immunized with a prime-boost regimen) showed the highest levels of T-cell proliferative responses against SIV Gag proteins ($P < 0.01$). The mean SIs for each of the five groups were 3.6 ± 1.2, 0.8 ± 0.4, 9.3 ± 2.3, 4.4 ± 1.5, and 3.4 ± 0.96, respectively (SIV Gag protein-stimulated group in Fig. 3A). Depletion of the $CD4^+$- or $CD4^+$ $CD8^+$-T-cell fraction from group 3's splenocytes dramatically reduced the proliferative responses to <10% (Fig. 3B). In contrast, the proliferative activity was not affected by the depletion of the $CD8^+$ fraction from the cell suspensions.

The splenocytes from the mice in group 3 also exhibited the highest proliferative responses against pooled peptides spanning the full-length SIV Gag protein (Fig. 3). Among the three positive peptide pools (4, 6, and 7), reactivity to pool 6 was the highest, with a mean SI of 5.4 ± 2.8 (Fig. 3A). Peptides in this pool correspond to the SIV p27 region and encode an SIV-specific $CD4^+$-T-cell epitope, which is recognized by the $H\text{-}2^d$ allele. Splenocytes from mice in control group 2 were not reactive with any of the SIV antigens (SI < 1.0).

Immunization with the prime-boost regimen induces Gag-specific Th1-type responses. To further characterize the type

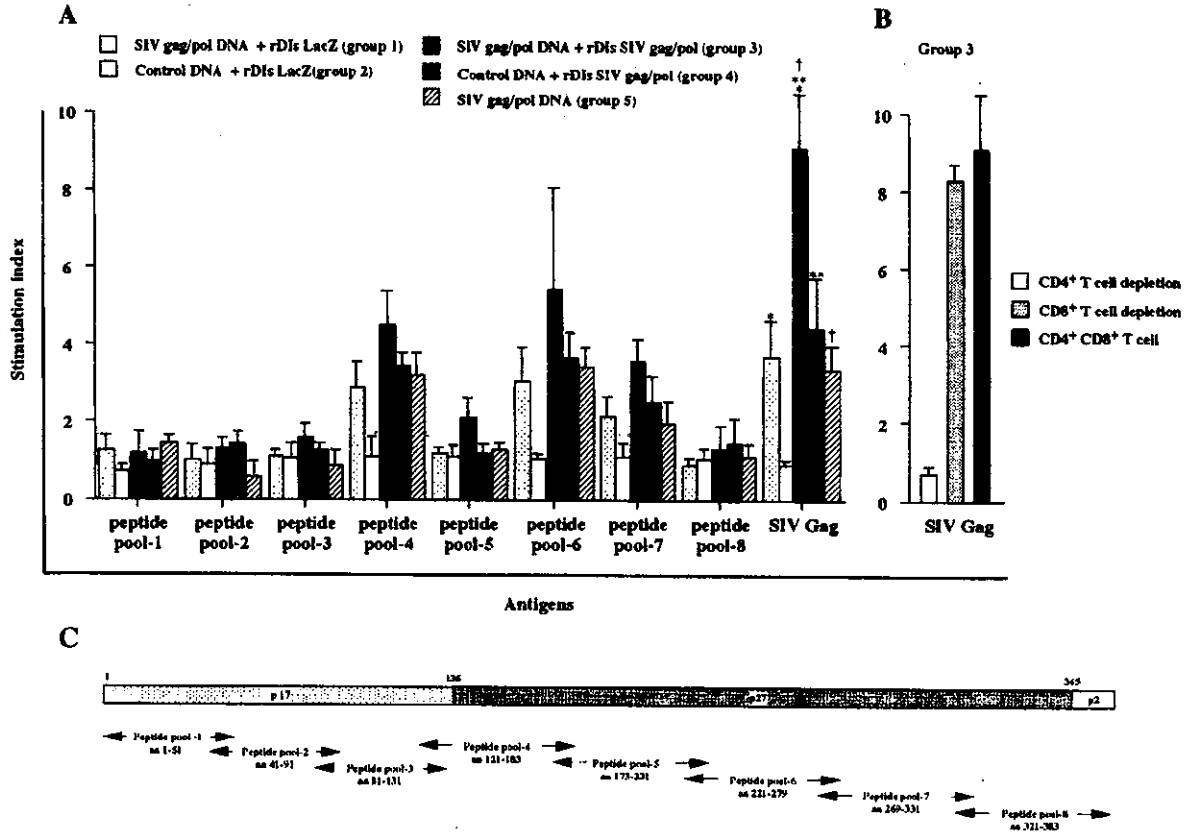

FIG. 3. Induction of SIV Gag-specific lymphocyte proliferative responses. (A) Spleen cells were cultured in the presence or absence of SIV Gag antigens, and [^{3}H]thymidine incorporation was measured as described in Materials and Methods. Proliferative responses are expressed as the SI. (B) Aliquots of spleen cells from mice vaccinated with the prime-boost regimen were depleted of either the $CD4^+$-T-cell, the $CD8^+$-T-cell, or the $CD4^+$ $CD8^+$-T-cell subpopulation prior to measuring SIV Gag-specific proliferative responses (C). Set of 15-mer overlapping peptides spanning the full-length Gag protein of SIV_{mac239}. The peptides were grouped into eight pools to evaluate cell-mediated immune responses. Values indicated by the single asterisk, double asterisks, and dagger symbol all showed a P value of <0.01, which were compared to give the indicated P values between groups 1 and 3, groups 3 and 4, and groups 3 and 5, respectively.

of immune responses induced by a prime-boost vaccination with SIV*gag/pol* DNA and rDIsSIV*gag/pol*, efforts were made to identify distinct antigen-specific $CD4^+$-T-helper-cell subsets. $CD4^+$ T cells were isolated from splenocytes and restimulated in vitro with purified SIV Gag protein. Culture supernatants were then examined for evidence of antigen-specific Th1- or Th2-type cytokine secretion. $CD4^+$ T cells from the immunized mice in group 3 (prime-boost regimen) generated the highest levels of Th1 cytokines, including IFN-γ and IL-2, whereas no evidence was found for the secretion of Th2 cytokines such as IL-4, IL-5, IL-6, or IL-10 (Fig. 4). The levels of cytokines generated by $CD4^+$ T cells from mice belonging to control group 2 were undetectable. These results demonstrate that priming with SIV*gag/pol* DNA, followed by boosting with rDIsSIV*gag/pol*, effectively induces predominantly Th1-type cytokine production in mice.

Characteristics of SIV-specific immunities in immunized animals at virus challenge. The induction of antigen-specific IFN-γ secretion and CTL was also evaluated in the immunized mice. ELISPOT assays were used to measure the number of SIV-specific SFC secreting IFN-γ in splenocytes from the immunized mice in each group (Fig. 5). Cells were restimulated in vitro with either SIV Gag p27 protein or pooled peptides spanning the full-length SIV Gag. SIV Gag-specific SFC were induced in mice receiving SIV*gag/pol* DNA alone, rDIsSIV*gag/pol* alone, or the combined prime-boost regimen. The number of SFC was higher in spleen cells from mice of group 3 immunized with the prime-boost regimen when stimulated with SIV Gag p27 than with Gag protein (735 ± 124 SFC per 10^6 splenocytes) than in those of mice immunized with either SIV*gag/pol* DNA or rDIsSIV*gag/pol* alone ($P < 0.01$). Stimulation of spleen cells of group 3 with whole Gag showed a stronger response than with Gag peptide pools 3, 5, and 8, with ELISPOT activities of 582 ± 121, 532 ± 117, and 394 ± 85 SFC per 10^6 splenocytes, respectively.

To determine whether the prime-boost regimen was able to induce antigen-specific CTL, ^{51}Cr-release assays were performed 1 week after the final inoculation. Spleen cells were isolated and restimulated in vitro for 7 days with each of eight different peptide pools. Cytotoxic activity was evaluated at effector/target (E:T) ratios of 100:1 to 12.5:1. SIV Gag-specific CTL activity was detected after stimulation with peptide pools 3, 5, and 8 (Fig. 6). The highest specific activity was induced by the prime-boost regimen after restimulation with peptide pool

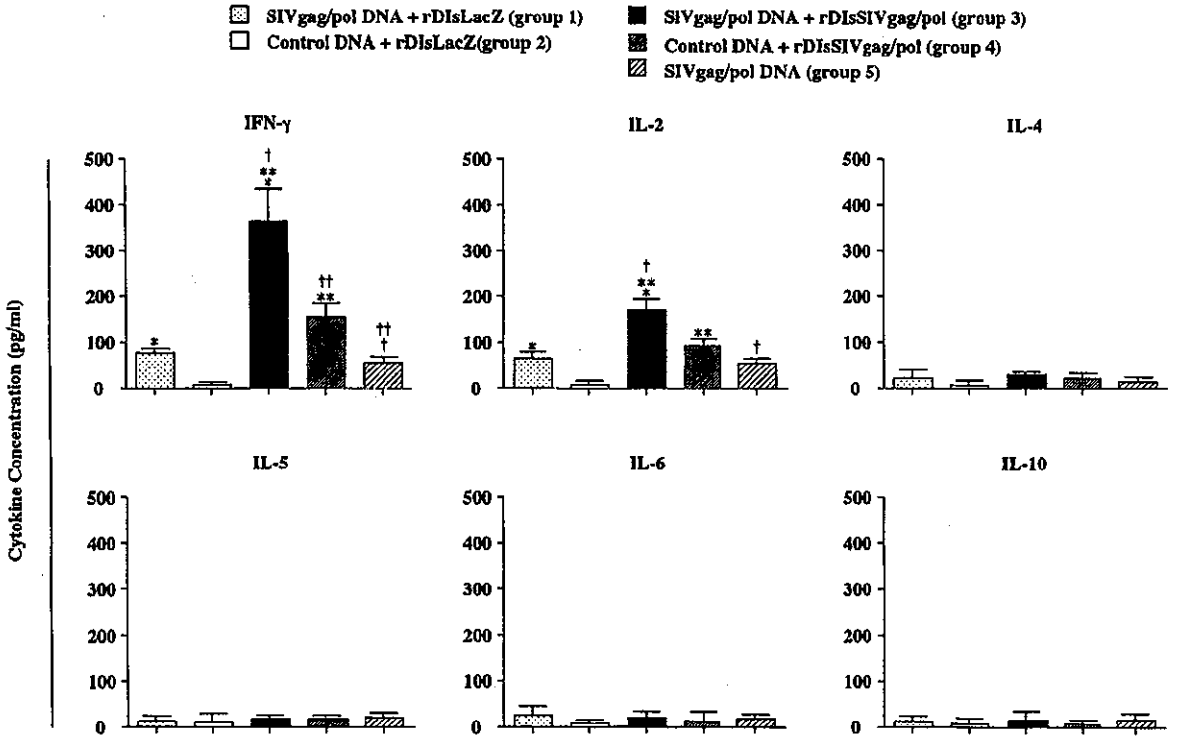

FIG. 4. In vitro production of IFN-γ, IL-2, IL-4, IL-5, IL-6, and IL-10 from spleen cells of immunized mice. Spleen cells were stimulated with recombinant SIV Gag protein, and secreted cytokines were quantified by cytokine-specific ELISA. The single asterisk, double asterisks, and dagger symbol all indicate a P value of <0.01, and the double dagger symbol (‡) indicates a P value <0.05, compared to give the indicated P values between groups 1 and 3, groups 3 and 4, groups 3 and 5, and groups 4 and 5, respectively.

3 (60% ± 11% specific lysis at an E:T ratio of 100:1). In contrast, spleen cells restimulated with peptides from pools 1, 2, 4, 6, and 7 showed little (<20%) or no specific lysis, as did cells from animals vaccinated with control DNA. CTL activity induction paralleled that of IFN-γ-secreting SFC (Fig. 5) and was dependent on the choice of immunizing and restimulating antigens.

We studied whether this regimen also enhances specific antibody responses (Fig. 7). Three consecutive inoculations of SIV*gag/pol* DNA, followed by two of rDIsLacZ in group 1 and five consecutive SIV*gag/pol* DNA vaccinations in group 5, showed low levels of Gag-specific antibody responses, with ELISA titers of <120 throughout the immunization period. Group 4 animals receiving three control DNAs, followed by two rDIsSIV*gag/pol* vaccinations, and group 5 animals with the prime-boost vaccination also elicited low levels of SIV Gag-specific antibody responses with titers of <180, showing that the induction of SIV Gag-specific humoral responses are very low in these vaccination regimens.

Elicitation of positive immunity by the prime-boost vaccine regimen against challenge with the wild-type SIV*gag/pol*-expressing vaccinia virus. Seven days after final vaccination, immunized mice were challenged i.p. with 10^7 PFU of the wild-type vaccinia virus strain vv9019, which expressed SIV*gag/pol*. At 6 days after the viral challenge, the mice were sacrificed, and ovaries were harvested to determine the viral load of the challenge virus in the organs. Among the five vaccinated groups, group 3 mice immunized by the prime-boost vaccine regimen showed a striking inhibition of viral infection into ovaries, with a fold reduction as high as 322 ± 48 (dark column in Fig. 8). The mice immunized with other regimens of group 1, 2, 4, or 5 (Fig. 2) showed fold reductions of 52 ± 23, 4 ± 5, 112 ± 21, and 41 ± 10, respectively, in the virus titer. By comparing the different groups with each other and with naive mice, we defined that the prime-boost vaccine group 3 showed statistically the most significant reduction of the viral load of the wild-type virus in tissues ($P < 0.01$). However, although vaccinated with SIV*gag/pol* prime and rDIsSIV*gag/pol* boost, group 3 exhibited no protection when challenged with wild-type vaccinia virus. The comparable vaccine efficacy to these animal groups were achieved by a second-series immunization experiment with 50 more animals (Fig. 9). These results suggest that vaccination with an SIV*gag/pol* DNA prime, followed by a rDIsSIV*gag/pol* boost, leads to a protective immunity against challenge with wild-type recombinant vaccinia virus in the immunized animals.

Gag-specific responses dominate in the positive immunity induced by the prime-boost regimen at the time of challenge. We then studied the immune responses by differentiating the gag-, pol-, and vaccinia virus antigen-specific responses in respective antigen-specific ELISPOT assays (Fig. 10). At the time of vv9019 challenge, we defined not only SIV Gag- but also SIV Pol-specific T-cell responses in immunized animals in groups 1, 3, 4 and 5. However, the Gag response was remarkably higher than the Pol response in group 3 of the prime-boost regimen ($P < 0.01$) with a less pronounced but similar ten-

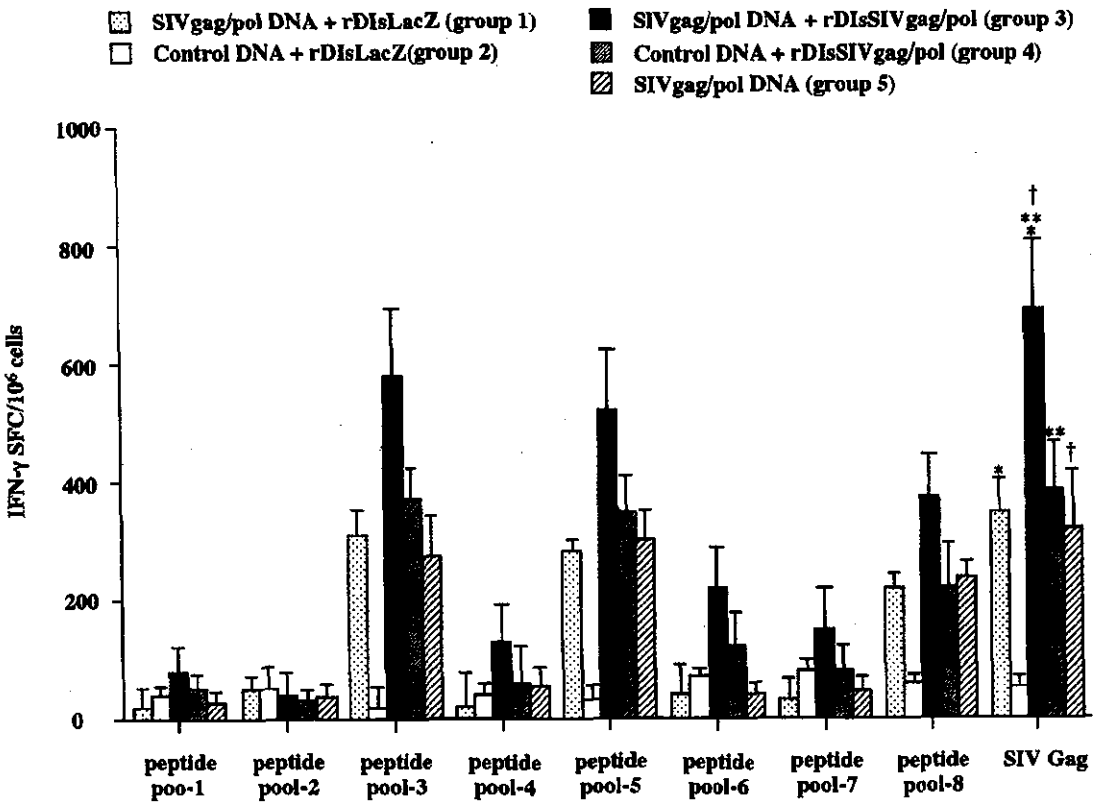

FIG. 5. Frequency of SIV Gag-specific IFN-γ-producing cells in immunized mice. Spleen cells were stimulated with either SIV Gag protein or pooled SIV Gag peptides. IFN-γ-producing cells were detected by IFN-γ-specific ELISPOT assays, and data are expressed as the number of SFC per 10^6 splenocytes. The single asterisk, double asterisks, and dagger symbol all showed a P value of <0.01, which were compared to give the indicated P values between groups 1 and 3, groups 3 and 4, and groups 3 and 5, respectively.

dency seen in groups 1, 4, and 5 ($P < 0.01$). In contrast, vaccinia virus antigen-specific ELISPOT activities were all extremely low, and the positive spots numbered fewer than 80 per million of spleen cells among vaccinia virus- and recombinant vaccinia virus-inoculated groups 2, 3, and 4. The results were not significantly different among the three groups of animals tested, suggesting that the very low levels of vaccinia virus antigen-specific immunity do not significantly contribute to the induction of positive immunity in the prime-boost regimen. These findings demonstrate that the SIVGag-specific T-cell responses dominate in the elicitation of positive immunity induced by the prime-boost vaccine regimen with vaccines expressing SIV*gag/pol*.

DISCUSSION

Previously, we demonstrated that DNA-based vaccination results in the induction of virus-specific immunity to several viral pathogens, including HIV-1 (3, 47, 48, 53, 64, 76). Furthermore, we recently established a system to express HIV-1 genes by inserting them into a deleted region of the attenuated vaccinia virus strain, DIs (25, 30, 71). Like the parental DIs strain, the recombinant DIs-HIV was shown to be completely replication deficient in mammalian cells. Moreover, the expression of SIVGag was sufficient to elicit positive immunity against pathogenic viral challenge in a SHIV-macaque model (26). In the present study, the prime-boost regimen with HIV-DNA and rDIs-HIV clearly enhanced the protective efficacy over that of rDIs-HIV alone or HIV-DNA alone. Although it is not possible to directly compare protective efficacy among different vector-based vaccine models, recombinant vaccinia virus strains (including MVA) (69), a substrain of Copenhagen (NYVAC) (73), and recombinant adenovirus-HIV strains (68), our results appear to be as effective for obtaining protective immunity as those achieved with vector-based vaccines. Taken together, these results suggest that a combination regimen of DNA and rDIs might be used as a safe and effective vaccine.

In the present study, we addressed whether a prime-boost regimen consisting of a plasmid DNA prime and rDIs boost could promote a strong Th1-type immune response capable of affecting the outcome of experimental challenge. It has been proposed that Th1-type responses are associated with protection against infection, including HIV-1 infection and AIDS. Individuals who control HIV-1 viremia in the absence of antiviral therapy respond to HIV-1 Gag protein and its helper epitopes with a Th1-like response, producing IFN-γ and β-chemokines (58). Moreover, a shift from Th1- to Th2-dominant cytokine production occurs during the course of HIV-1 disease progression (36, 39, 65), suggesting that the cytokine profile may be indicative of a T helper phenotype and represent a

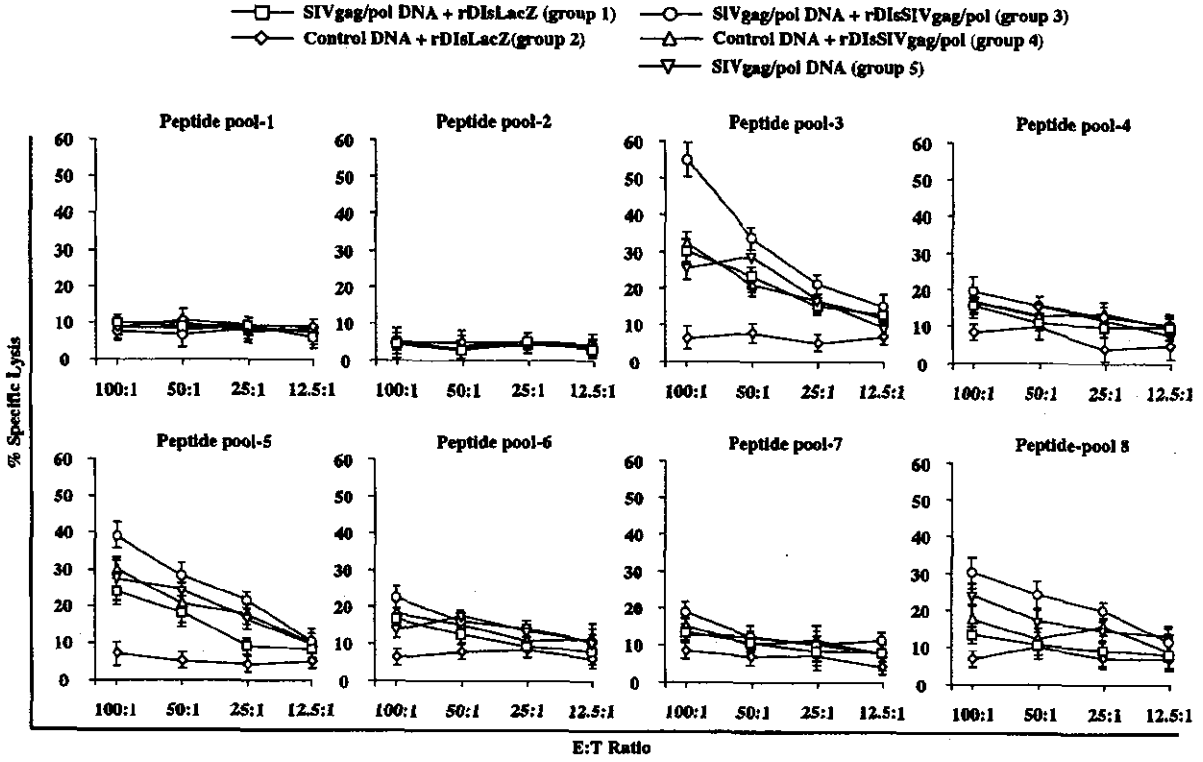

FIG. 6. Induction of SIV Gag-specific CTL in immunized mice. Spleen cells were stimulated with pooled SIV Gag peptides and tested in ^{51}Cr release assays with peptide pulsed M12.4.4 cells as targets cells at E:T ratios ranging from 100:1 to 12.5:1.

response to infection. Our results demonstrate that Th1-type cytokines, including IL-2 and IFN-γ, are secreted by $CD4^+$ T cells from mice immunized with a prime-boost regimen targeting the SIV*gag/pol* region. These Th1-type responses were associated with SIV Gag protein- or peptide-specific SFC activity

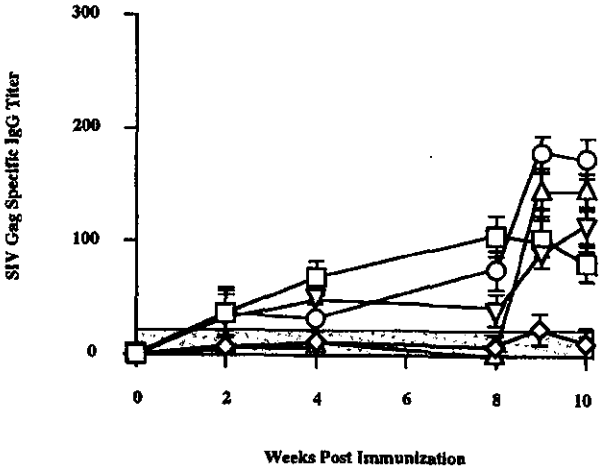

FIG. 7. Kinetics of binding antibody titer specific for SIV Gag in mice. The endpoint titers of immune sera were measured by the SIV Gag p27 antigen-ELISA at each time point. Bars represent the mean ± the SD value of four independent experiments. Symbols: □, SIV*gag/pol* DNA + rDIsLacZ (group 1); ◇, control DNA + rDIsLacZ (group 2); ○, SIV*gag/pol* DNA + rDIsSIV*gag/pol* (group 3); △, control DNA + rDIsSIV*gag/pol* (group 4); ▽, SIV*gag/pol* DNA (group 5).

and $CD8^+$ CTL. These observations are encouraging in light of the hypothesis that Th1-mediated immunity is associated with resistance to HIV infection and virus suppression (6, 31). In vitro restimulation of splenocytes from mice immunized with the prime-boost regimen generated high levels of Th1 cytokines, such as IL-2 (>100 pg/ml) and IFN-γ (>300 pg/ml), and lower levels of Th2 cytokines, such as IL-4, IL-5, IL-6, and IL-10 (<30 pg/ml). In contrast, immunization with either SIV*gag/pol* DNA or rDIsSIV*gag/pol* alone led to lower levels of Th1-type cytokine production, suggesting that the prime-boost regimen is superior for the induction of SIV Gag-specific Th1-type T-cell responses.

Having observed an induction of SIV Gag-specific Th1-type responses in mice after immunization with the prime-boost regimen, we also detected significant levels of virus-specific proliferative responses in spleen cells from the immunized animals. Fractionation of the spleen cell population revealed that the SIV-specific lymphocyte responses were mediated by $CD4^+$ T cells. Several reports have demonstrated that HIV-1-specific $CD4^+$ T-cell proliferation inversely correlates with disease progression in infected individuals (37, 78). Moreover, HIV-1 Gag p24-specific $CD4^+$-lymphocyte proliferation has been shown to be inversely correlated with the HIV-1 load in plasma (58, 59). Although SIV-specific T-cell proliferative responses were induced in mice immunized with either SIV*gag/pol* DNA or rDISSIV*gag/pol* alone, the SI was generally not as high as that obtained by the combined prime-boost regimen. Our data showing the induction of $CD4^+$-T-cell proliferative responses to SIV Gag in mice immunized with the

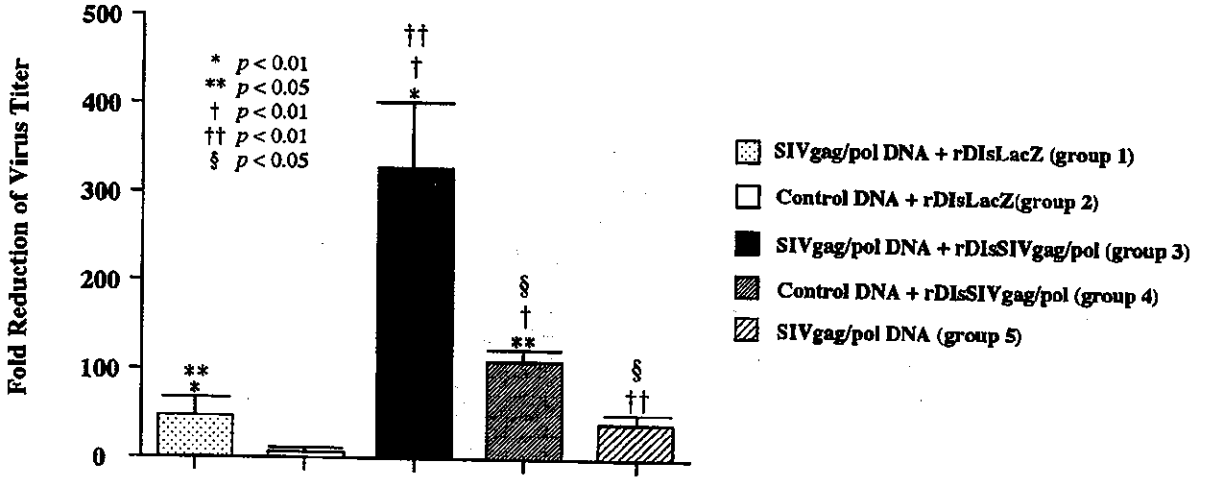

FIG. 8. The prime-boost vaccine regimen augmented protective immune responses. The animals immunized with five different strategies were challenged i.p. with 10^7 PFU of wild-type vaccinia virus strain vv9019 recombinant expressing SIV*gag/pol*. The bar shows the fold reduction of vaccinia virus titer in the ovaries of vaccinated mice versus naive mice. Bars show the geometric mean values of four mice per group.

prime-boost regimen suggests this vaccine approach may be effective at inducing strong virus-specific $CD4^+$-T-cell responses capable of controlling viral load in the immunized animals.

The importance of T helper responses is highlighted in reports that antigen-specific $CD4^+$ T helper cells may promote CTL activity either by a CD4-antigen-presenting cell-CD8 pathway and IL-2 secretion (18, 58, 79) or by an increased production of antiviral cytokines and chemokines. Furthermore, $CD4^+$ T cells promote other types of cell-mediated immunity, including activation of macrophages and cytokine secretion, which may also contribute to the control of HIV-1 and other intracellular pathogens. Recent reports have documented that a vaccine regimen consisting of a DNA prime and a recombinant poxvirus boost generates pathogen-specific protective immune responses (2). The protective role of CTL is also well documented in HIV-1 infection (9, 28, 41, 44, 50, 52, 56, 60, 61, 62), and the induction of an HIV-1-specific CTL population is considered an important goal for most current vaccine strategies. HIV-1 Gag-specific $CD8^+$ cytolysis has been highly correlated with IFN-γ synthesis by $CD8^+$ spleen T cells (49). In the present study, the prime-boost regimen induced significant levels of SIV Gag-specific IFN-γ-producing cells (>700 SFC/10^6 splenocytes). These responses were higher than those induced by immunization with either SIV*gag/pol* DNA or rDIsSIV*gag/pol* alone.

In conclusion, our data show that a new vaccine regimen consisting of SIV*gag/pol* DNA priming and rDIsSIV*gag/pol* boosting induces strong SIV Gag-specific and Th1-type cellular immune responses, which were associated with the control of viral challenge. Since the magnitude and phenotype of the induced immunity are believed to be associated with protection against viral infection and disease progression, this new priming-boosting vaccine regimen may be useful for the develop-

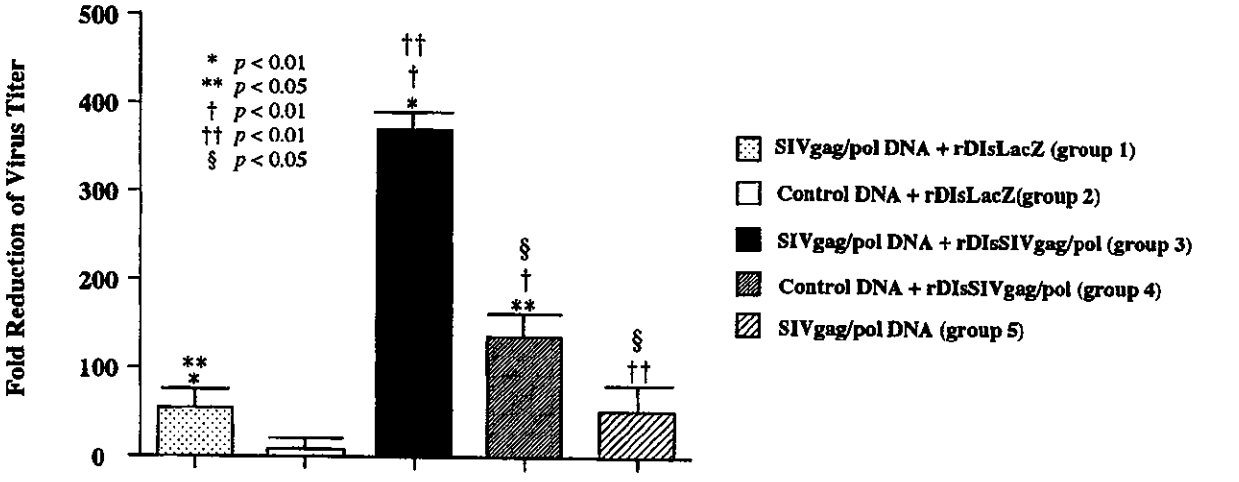

FIG. 9. A second series immunization with the prime-boost regimen resulted in a similar augmentation of protective immune responses. Fifty animals were divided into five groups of 10 animals each, and the animales were immunized by the five different strategies, respectively, described in Fig. 8.

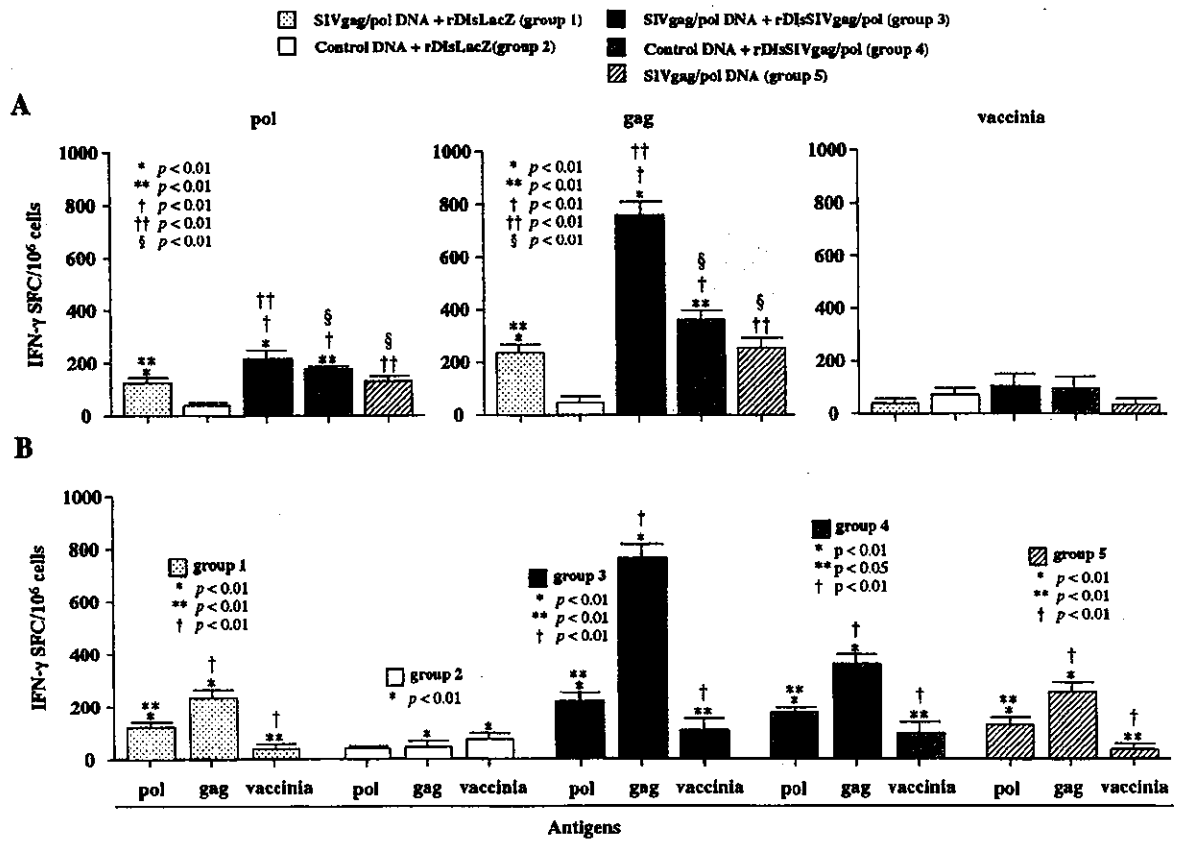

FIG. 10. Comparison of SIV*gag*-, SIV*pol*-, and vaccinia virus antigen-specific immunities in animals at the time of challenge. ELISPOT activities in vaccinated animals with different strategies. Each group of animals was immunized with different strategies and antigen-specific T-cell responses were analyzed by differentiating the SIV*gag*-, SIV*pol*-, and vaccinia virus antigen-specificities at the time of challenge infection by using protein antigen-specific ELISPOT assays. (A) SIV*gag*-, SIV*pol*-, and vaccinia virus antigen-specific analyses; (B) vaccine strategy-specific analysis of each group of five.

ment of an HIV-1 candidate vaccine. This strategy will be further evaluated to determine its efficacy against viral challenge in a nonhuman primate model.

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Vaccination of Rhesus Macaques with Recombinant *Mycobacterium bovis* Bacillus Calmette-Guérin Env V3 Elicits Neutralizing Antibody-Mediated Protection against Simian-Human Immunodeficiency Virus with a Homologous but Not a Heterologous V3 Motif

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Although the correlates of vaccine-induced protection against human immunodeficiency virus type 1 (HIV-1) are not fully known, it is presumed that neutralizing antibodies (NAb) play a role in controlling virus infection. In this study, we examined immune responses elicited in rhesus macaques following vaccination with recombinant *Mycobacterium bovis* bacillus Calmette-Guérin expressing an HIV-1 Env V3 antigen (rBCG Env V3). We also determined the effect of vaccination on protection against challenge with either a simian-human immunodeficiency virus (SHIV-MN) or a highly pathogenic SHIV strain (SHIV-89.6PD). Immunization with rBCG Env V3 elicited significant levels of NAb for the 24 weeks tested that were predominantly HIV-1 type specific. Sera from the immunized macaques neutralized primary HIV-1 isolates in vitro, including HIV-$1_{BZ167/X4}$, HIV-$1_{SF2/X4}$, HIV-$1_{CI2/X4}$, and, to a lesser extent, HIV-$1_{MNp/X4}$, all of which contain a V3 sequence homologous to that of rBCG Env V3. In contrast, neutralization was not observed against HIV-$1_{SF33/X4}$, which has a heterologous V3 sequence, nor was it found against primary HIV-1 R5 isolates from either clade A or B. Furthermore, the viral load in the vaccinated macaques was significantly reduced following low-dose challenge with SHIV-MN, and early plasma viremia was markedly decreased after high-dose SHIV-MN challenge. In contrast, replication of pathogenic SHIV-89.6PD was not affected by vaccination in any of the macaques. Thus, we have shown that immunization with an rBCG Env V3 vaccine elicits a strong, type-specific V3 NAb response in rhesus macaques. While this response was not sufficient to provide protection against a pathogenic SHIV challenge, it was able to significantly reduce the viral load in macaques following challenge with a nonpathogenic SHIV. These observations suggest that rBCG vectors have the potential to deliver an appropriate virus immunogen for desirable immune elicitations.

Development of a preventive vaccine against human immunodeficiency virus type 1 (HIV-1) is urgently needed to control the spread of the virus worldwide. Although the immunological parameters that correlate with protective immunity against natural infection with HIV-1 are not fully known, it is assumed that a preventive vaccine must elicit potent, broadly reactive immunity against divergent strains of HIV-1 (25, 36, 42). Several recent studies have demonstrated that induction of virus-specific T-cell responses can confer protective immunity in nonhuman primate models, and these responses may also play a role in controlling HIV-1 replication in humans (6, 18, 19, 31, 33, 34, 38, 45, 48). Vaccine constructs containing viral *env* genes, in addition to *gag* and *pol*, have been shown to effectively control replication of challenge viruses (2, 5, 10), suggesting that neutralizing antibody (NAb) responses might also contribute to protection against pathogenic infection or disease progression. Passive transfer of serum immunoglobulin from chimpanzees experimentally infected with several different HIV-1 isolates has been shown to block the establishment of a simian immunodeficiency virus (SIV)-HIV chimeric simian-human immunodeficiency virus (SHIV) infection in pig-tailed macaques (37, 46). It is not known, however, whether vaccines that actively elicit a potent NAb response can provide protection in nonhuman primates challenged with SHIV.

Previously, we demonstrated that recombinant *Mycobacterium bovis* bacillus Calmette-Guérin (rBCG), which secretes a chimeric protein consisting of the V3-neutralizing epitope of HIV-1 and α-antigen (rBCG Env V3), can induce HIV-1-specific NAb in a small-animal model (9, 15, 16). BCG was selected as a vaccine vehicle because it has several characteristics that are considered efficacious for developing a candidate

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HIV-1 vaccine (1, 49), including the ability to induce long-lasting immune responses (7). It is generally accepted that a candidate vaccine against HIV-1 must also be easily administered and affordable in developing countries, and it must be compatible with other commonly administered vaccines (35). If effective, a BCG-based recombinant HIV-1 (rBCG–HIV-1) vaccine would fulfill many of these critical requirements.

Results using other vaccine modalities, in particular, live attenuated SIV vaccines, have raised concerns about the potential for reversion to pathogenicity (3, 4), suggesting that many SIV strains may be potentially virulent. In this study, we used two distinct strains of challenge virus: SHIV-MN (29), which contains V3 sequences homologous to rBCG Env V3, and SHIV-89.6PD (12, 20, 28, 41), which is heterologous in the V3 region and highly pathogenic. We examined whether vaccination with rBCG Env V3 could effectively elicit NAb responses in rhesus macaques and whether it might effectively induce protective immunity against challenge with either SHIV-MN or SHIV-89.6PD.

MATERIALS AND METHODS

Animals. The macaques (*Macaca mulatta*) used in this study originated from China and were purchased through Japan SLC Ltd., Shizuoka, Japan. The animals were maintained according to standard operating procedures established for the evaluation of human vaccines at the Tsukuba Primate Center, National Institute of Infectious Diseases, Tsukuba, Ibaragi, Japan. The study was conducted in the P3 facility for monkeys in the Murayama Branch, National Institute of Infectious Diseases, Musashimurayama, Tokyo, Japan, and in accordance with requirements specified in the laboratory biosafety manual of the World Health Organization.

Construction of the rBCG Env V3 immunogen. rBCG substrain Tokyo was produced by *transfection of* BCG-Tokyo 172 cells with plasmid pSO246 as described previously (21, 22, 30). The XhoI site of this plasmid was used to insert a mycobacterial codon-optimized DNA fragment encoding 19 amino acids of the Japanese HIV-1 V3 consensus sequence (NTRKSIHIGPGRAFYATGS), which has a neutralization sequence identical to that of HIV-1_{MN} (16, 23, 39, 52). The resulting rBCG vector was designated rBCG Env V3. By semiquantitation of a chimeric protein consisting of the V3 peptides and α-K protein (9), the concentration of the secreted protein was estimated to range *from 1 to 3 μg/ml in the* culture filtrate of rBCG Env V3 (data not shown).

Viruses. Viruses used in challenge experiments were kindly provided by Y. Lu, Harvard AIDS Institute, Cambridge, Mass. The SHIV-MN virus stock was prepared in concanavalin A-activated macaque peripheral blood mononuclear cells (PBMC) from normal animals, and the amount of virus was quantified by SIV p27 antigen enzyme-linked immunosorbent assay (ELISA) (Coulter Co., Hialeah, Fla.). The tissue culture infective dose (TCID) of the stock was measured on CEMx174 cells (AIDS Research and Reference Reagent Program, National Institutes of Health, Rockville, Md.). Stocks of HIV-1_{MN} and HIV-1_{IIIB} (AIDS Research and Reference Reagent Program) were prepared by propagating 100 50% TCID ($TCID_{50}$) of each virus in phytohemagglutinin-activated normal human PBMC, as described previously (17). The primary isolate, HIV-1_{MNp}, was *kindly provided* by J. Sullivan, University of Massachusetts Medical School, Worcester, Mass. All other viruses were obtained from the AIDS Research and Reference Reagent Program. Cell-free virus stocks were stored at −130°C until they were used.

V3-specific ELISA. HIV-1 V3 peptide-based ELISAs were used for titration and quantification of serum antibodies for detection as described previously (14). In brief, 96-well ELISA plates (MaxiSorp; Nunc A/S, Roskilde, Denmark) were *coated with 100 μl of peptide MN (DKRIHIGPGRAFYTT)*/well in 50 mM carbonate buffer (pH 9.3) at 5 μg/ml overnight at 4°C. The wells were washed and treated with 5% nonfat milk in phosphate-buffered saline for 1 h at 37°C. Duplicate samples containing either control or test macaque serum at appropriate dilutions were then added at 100 μl/well, and the plates were incubated for 1 h at 37°C. The wells were washed and incubated with a detection antibody solution consisting of peroxidase-conjugated goat anti-monkey immunoglobulin G (IgG) antibody (EY laboratories Inc., San Mateo, Calif.) at *100 μl/well for 1 h* at 37°C. After final washes with 0.05% Tween-20–phosphate-buffered saline (PBST), peroxidase substrate was added, and the reaction was stopped by the addition of 0.5 M H_2SO_4.

IFN-γ ELISPOT assay. Enzyme-linked immunospot (ELISPOT) assays were performed using the method developed by Mothe and Watkins of the Wisconsin University Primate Center and *described elsewhere* (18, 33). *In brief*, 96-well flat-bottom plates (U-CyTech-BV, Utrecht, The Netherlands) were coated with anti-gamma interferon (IFN-γ) monoclonal antibody before being washed with PBST and blocked with bovine serum albumin. Freshly isolated PBMC were mixed with either concanavalin A or 2 μM V3 peptide and were then incubated for 16 h at 37°C in 5% CO_2 in anti-IFN-γ-coated plates. Once the plates had been washed, rabbit anti-IFN-γ polyclonal biotinylated detector antibodies were added, and the plates were incubated. Gold-labeled anti-biotin *IgG solution* (U-CyTech-BV) was added to the plates after they were washed with PBST. The plates were then incubated for 1 h at 37°C. Developed wells were imaged, and spot-forming cells (SFC) were counted using the KS ELISPOT compact system (Carl Zeiss, Oberkochen, Germany). An SFC was defined as a large black spot with a fuzzy border (33).

In vitro virus neutralization assays. GHOST cell neutralization assays were performed as previously described (8). Briefly, GHOST cells expressing either CXCR4 or CCR5 were used as targets for HIV-1 infection (50, 54). The cells were analyzed by FACSCalibur flow cytometry (Becton Dickinson, San Jose, Calif.), and 15,000 events were scored. The mean number of fluorescent GHOST cells determined from negative controls plus 2 standard deviations was considered the cutoff for a positive sample. Purified human immunoglobulin (Nihon Pharmaceutical Co., Tokyo, Japan) *and saline were included as additional* controls.

M8166 cell-based virus neutralization assays were also performed as described previously (16, 47). In brief, the in vitro neutralization activity of purified macaque IgG was determined using 100 $TCID_{50}$ of either HIV-1_{MN} or SHIV-MN in cultures of M8166 cells. The results were compared with parallel cultures to which preimmune serum IgG was added. Neutralization was expressed as percent inhibition of HIV-1 p24 or SIV p27 antigen production in the culture supernatants. Purified normal macaque *IgG was used as a control.*

Quantification of cell-associated viral load. Levels of cell-associated virus were quantified by limiting dilution of PBMC (from 10^6 to 1 cells), and the virus was cocultured with M8166 cells as described previously (17). Virus released into the culture supernatant was measured by SIV p27 antigen ELISA (Coulter). The smallest number of PBMC required to produce a positive culture was considered the end point, and the titer of infectious virus was expressed as $TCID_{50}$ per 10^6 PBMC.

PCR detection of proviral HIV-1 infection of rhesus macaques. *PBMC* with SHIV were detected by DNA PCR using a primer pair that spans the C2-V3 sequence of HIV-1_{IIIB}, followed by Southern blotting with an SE1 probe, 5′-GCAGAAGAAGAGGTAGTAATTAGAT-3′ (nucleotides 7019 to 7043) (47). The positions of the oligonucleotides are numbered relative to the HIV-1_{HXB2} isolate in the ENTREZ database (National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Md.). Viral DNA was quantified by comparison with standards derived from 8E5/LAV cells, which contain one copy of HIV-1 proviral DNA per cell (AIDS Research and Reference Reagent Program).

Competitive PCR quantitation of SHIV RNA in plasma. Quantitative, competitive reverse transcription-PCR was performed using a competitor RNA and a DNA template as previously described (18, 32, 44). The detection limit of this *assay was 500 RNA copies/ml in monkey* plasma (18, 32).

Sequencing of HIV-1 Env C2-V3 sequence. To determine the sequence of the HIV-1 Env C2-V3 region, mRNA was extracted from stock virus and cDNA was synthesized using a Micro-FastTrack version 2.0 kit (Invitrogen, Carlsbad, Calif.) and a cDNA cycle kit (Invitrogen) according to the manufacturer's instructions. The PCR products were cloned into a pCR II vector with a dual promoter using a TA cloning kit (Invitrogen) (47). Sequence analysis was performed using a Big Dye terminator cycle-sequencing FS kit *(Perkin-Elmer, Foster City, Calif.) and auto*mated ABI 310 sequencer (Perkin-Elmer) with Sp6 and T7 sequence primers (Invitrogen). Sequence data were compared with published HIV-1 sequences in GenBank (National Center for Biotechnology Information, National Institutes of Health).

Statistical analysis. Calculations of the geometric mean ± standard deviation (SD) were carried out with a microcomputer. Significance was defined as a P value of <0.05.

RESULTS

Vaccination protocol. Twenty-four male rhesus macaques (R-01 through R-24) were enrolled in the study. Of these, 15 were subcutaneously immunized for 24 weeks with 10 mg of