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Vaccination with Attenuated Simian Immunodeficiency Virus by DNA Inoculation

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Delivering attenuated lentivirus vaccines as proviral DNA would be simple and inexpensive. Inoculation of macaques with wild-type simian immunodeficiency virus strain mac239 (SIV_{mac239}) DNA or SIV_{mac239} DNA containing a single deletion in the 3′ *nef*-long terminal repeat overlap region (*nef*/LTR) led to sustained SIV infections and AIDS. Injection of SIV_{mac239} DNA containing identical deletions in both the 5′ LTR and 3′ *nef*/LTR resulted in attenuated SIV infections and substantial protection against subsequent mucosal SIV_{mac251} challenge.

nef-deletion-containing live-attenuated simian immunodeficiency virus (SIV) vaccines have been shown to be efficacious in macaques (3). However, a proportion of macaques and humans become immunodeficient following infection with *nef*-deletion-containing SIV or human immunodeficiency virus type 1 (HIV-1) strains (1, 4, 13). Should modified attenuated lentivirus vaccines eventually prove safe (17), delivering such viruses to large numbers of people in developing countries raises logistical problems, including quality control and cold-chain issues. These difficulties would be overcome if an infection with an attenuated HIV-1 vaccine could be initiated via proviral plasmid DNA. Pathogenic lentiviral infection of cats following wild-type feline immunodeficiency virus DNA inoculation and of macaques following SIV_{mac239} DNA inoculation have been induced by the intramuscular administration of 50 to 500 μg of DNA (7, 14, 16, 21). Attenuating deletions in the 5′ long terminal repeat (LTR) (which drives the initial round of transcription) affect the initial expression of reporter genes in macaques and in human skin ex vivo (9) and could render the 5′-LTR-deletion-containing constructs noninfectious in vivo. The utility of proviral DNA in initiating an attenuated SIV infection was therefore studied in macaques.

Proviral SIV constructs with either a single deletion in the 3′ *nef*-LTR overlap region (*nef*/LTR) ($SIV_{sbbc\Delta3}$) analogous to the common deletion observed in HIV-1 strains isolated from the Sydney Blood Bank Cohort (SBBC) or an additional identical deletion in the 5′ LTR ($SIV_{sbbc\Delta3\Delta5}$) were engineered into a low-copy-number vector, pKP55, kindly provided by K. Peden (Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Md.) (Fig. 1). The distal half of the SIV_{mac239} provirus, 3′ to the *Sph*I site (position 6446, GenBank accession no. M33262), was cut with *Sph*I and *Eco*RI from plasmid p239SpE3′ (contributed to the National Institutes of Health AIDS reagent repository by R. Desrosiers) and ligated into the unique sites of the pKP55 vector (5, 12). A 105-bp in-frame *nef*/LTR deletion from position 9657 that re-

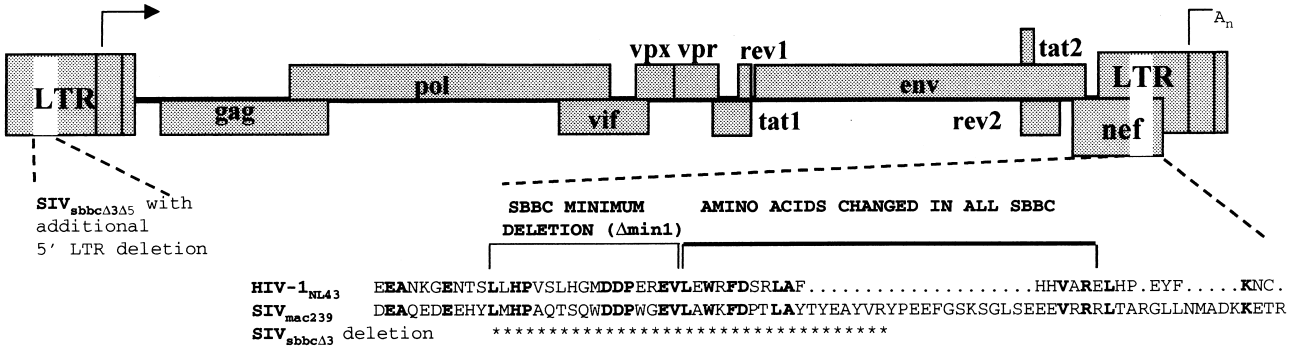

FIG. 1. SIV_{mac239}-based plasmids. Asterisks denote the 105-bp (35-amino-acid) deletion in the 3′ *nef*/LTR of construct $SIV_{sbbc\Delta3}$. A further, identical deletion in the 5′ LTR was made in $SIV_{sbbc\Delta3\Delta5}$. The deletion in SIV *nef*/LTR is analogous to most of the shared *nef*/LTR sequence deletions present in HIV-1 strains found in the SBBC (4); amino acids common to Nef in both $HIV\text{-}1_{NL4\text{-}3}$ and SIV_{mac239} are noted in bold.

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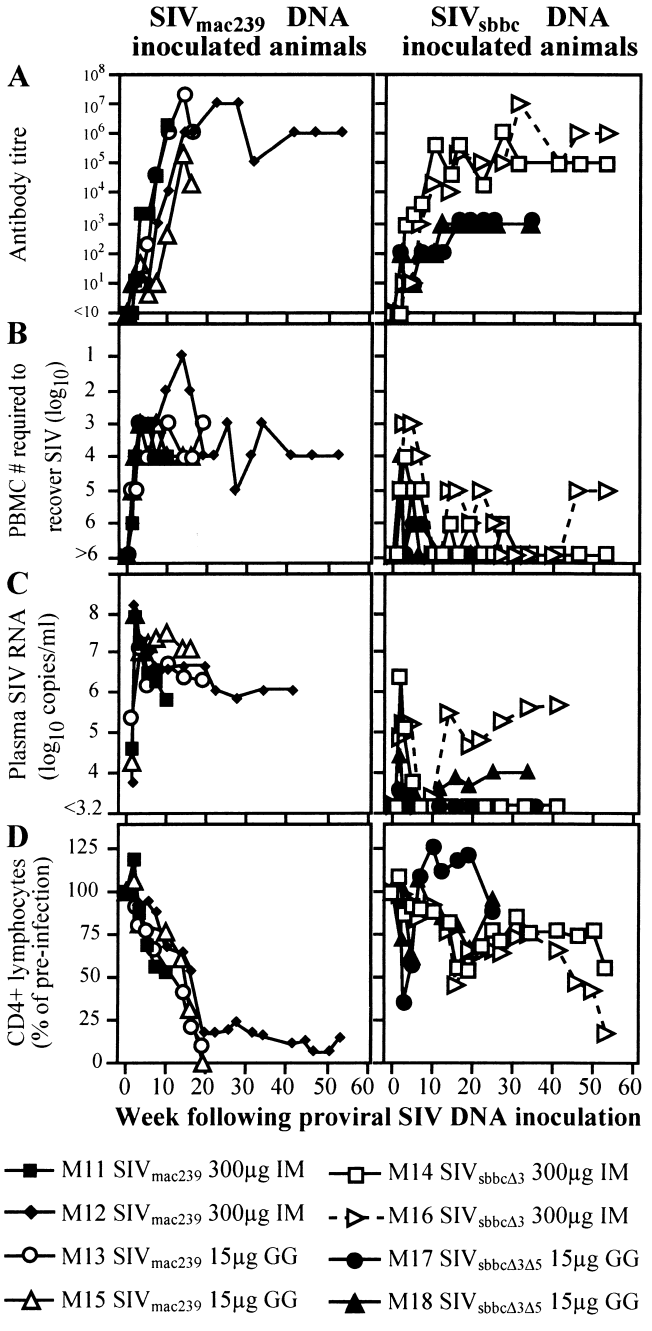

FIG. 2. SIV infection of macaques by administration of SIV proviral DNA. Plasmids, doses, and routes (GG, epidermally via a gene gun; IM, intramuscularly) are noted in the key. (A) SIV antibody response by endpoint dilution. (B) Quantification of cell-associated SIV. The lowest numbers of PBMC required to recover SIV are shown. For some cultures (M11, weeks 3 and 5; M12, weeks 3, 5, 7, 10, and 14; M13, weeks 3 and 10; M15, week 7; M16, weeks 3 and 5), SIV was recovered from the lowest cell dilution assayed. (C) Plasma SIV RNA levels. Plasma was assayed for SIV RNA by bDNA analysis, except M17 at weeks 10, 19, and 34 following inoculation and M18 at weeks 19 and 34 following inoculation, which were assayed by real-time RT-PCR. (D) $CD4^+$ lymphocyte proportions. Serial PBMC samples were assayed for the proportion of $CD4^+$ lymphocytes in the $CD2^+$ lymphocyte population, and these values were compared to mean preinoculation levels standardized to 100%.

moves U3 sequences always changed in or deleted from HIV-1 strains from the SBBC (4, 6) was engineered using a Quick Change mutagenesis kit (Stratagene, La Jolla, Calif.) with the oligonucleotide Δmin2F (CAGGAGGATGAGGAGCATTA TTACCCAGAAGAGTTTGGAAGC) and its reverse complement, Δmin2R, to make plasmid pKP-SIV-3′Δmin2. The 5′ half of SIV_{mac239} was cut from p239SpSp5′ with *Sph*I and cloned into the *Sph*I-linearized plasmids to make one plasmid with full-length SIV_{mac239} and one with a 3′ *nef*/LTR deletion (pSIVsbbcΔ3). To make pSIVsbbcΔ5Δ3, the 5′ LTR was deleted from position 194 of p239SpSp5′ using oligonucleotides Δmin2F and Δmin2R and cloned into pKP-SIV-3′Δmin2. The constructs were confirmed to be correct by cloning and sequencing. Transfection stocks of both wild-type and *nef*/LTR-deletion-containing SIV constructs grew equally well, as determined by a reverse transcriptase (RT) assay (19) in CEMx174 cells in vitro (data not shown). The *nef*/LTR-deletion-containing SIV_{sbbc} constructs did not express detectable Nef protein by immunoblotting (data not shown).

Eight pigtailed macaques were inoculated with wild-type or *nef*/LTR-deletion-containing SIV DNA constructs, delivered either intramuscularly (300 μg) or epidermally via gene gun (only 15 μg) (Fig. 2). All animals were shown to have seroconverted to HIV-2 antigens by Western blotting (not shown) and a particle agglutination assay (Serodia HIV-1/2; Fujirebio, Tokyo, Japan) (Fig. 2A). SIV was recovered from phytohemagglutinin- and interleukin-2-activated peripheral blood mononuclear cells (PBMC) of all inoculated animals by coculture with CEMx174 cells (Fig. 2B). The four SIV_{sbbc}-inoculated animals required more input PBMC ($>10^4$) to recover SIV than did the four animals receiving SIV_{mac239}. Plasma SIV RNA was measured either by bDNA analysis (Bayer Diagnostics, Emeryville, Calif.) or real-time RT-PCR as previously described (8). Both SIV RNA assays had a lower limit of detection of 1,500 copies/ml and had similar quantification levels. SIV_{mac239} DNA-inoculated macaques had higher peak levels of SIV RNA in plasma than SIV_{sbbc}-inoculated macaques (Fig. 2C). SIV_{mac239}-inoculated animals maintained SIV RNA levels of $>10^6$ copies/ml and were euthanized at weeks 11, 19, 20, and 53, showing SIV-associated coagulopathy, septicemia, and weight loss (two animals) with a marked decline in peripheral $CD4^+$ lymphocytes measured by flow cytometry (11) (Fig. 2D). No attenuation of virulence was observed in the gene gun-inoculated animals despite the low dose of DNA administered.

Macaques given the SIV_{sbbc} plasmids had SIV RNA levels that fell below the detection threshold by week 7 following inoculation and remained low or stable in three of the four animals, which also had normal $CD4^+$ lymphocyte levels (Fig. 2C and D). However, SIV RNA levels in macaque M16, inoculated with $SIV_{sbbcΔ3}$, rose to $>4 \times 10^5$ copies/ml by week 46, and this animal subsequently had a decline in $CD4^+$ lymphocyte numbers and was euthanized with weight loss at week 61. To determine the cause of the loss of $CD4^+$ lymphocytes in M16, nested-PCR analysis of SIV DNA from lysed PBMC was performed using oligonucleotide primers from positions 9129 to 9148 and 10016 to 10036 for the first round and primers from positions 9191 to 9208 and 9872 to 9890 for the nested round. Nested PCR amplified wild-type size SIV *nef*/LTR from week 3 following SIV inoculation and thereafter (Fig. 3, panel

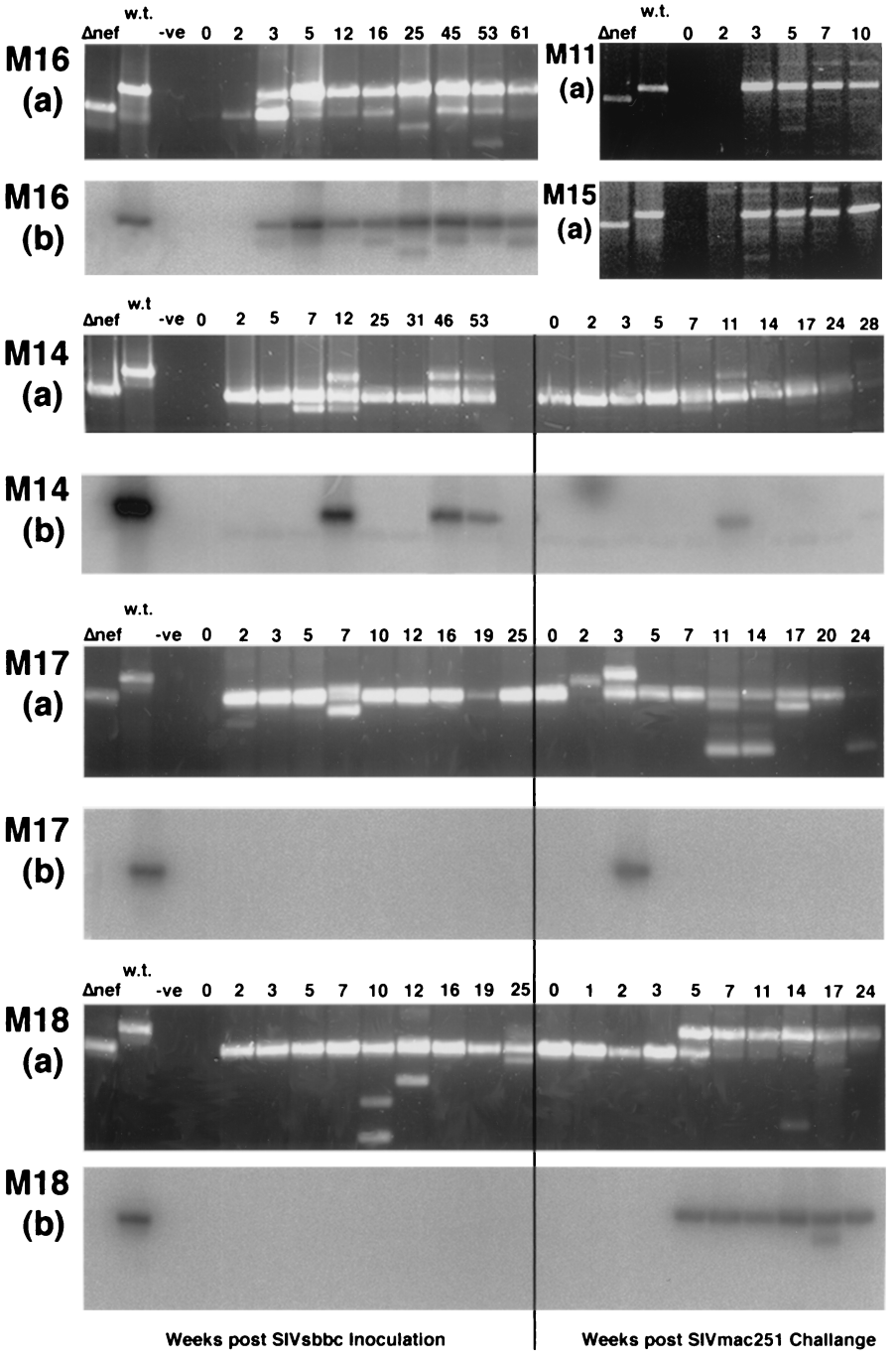

FIG. 3. PCR analysis of the SIV *nef*/LTR. (Panels a) Gel electrophoresis of PCR-amplified SIV proviral DNA isolated from macaque PBMC at various weeks following SIV plasmid DNA inoculation. As controls, *nef*/LTR PCR fragments were amplified from a control SIV_{mac239} plasmid bearing the wild-type (w.t.) sequence or the 105-bp SBBC strain deletion in SIV *nef*/LTR (Δnef). A wild-type-sized PCR product obtained after 25 cycles of PCR is shown from PBMC of two representative macaques, M11 and M15, that were inoculated with wild-type SIV_{mac239} plasmid DNA. Animals M14, M17, and M18 were challenged with SIV_{mac251} intrarectally at 68 weeks (M14) or 34 weeks (M17 and M18), and lanes with results for samples taken at these times are labeled week 0 to the right of the vertical line. (Panels b) Southern blotting of nested-PCR products from M16, M14, M17, and M18 probed with an oligonucleotide located within the *nef*/LTR deletion.

M16a). These wild-type-sized PCR fragments hybridized with a probe (oligonucleotide GTCATCCCACTGGGAAGTTTG AGCTG) internal to the *nef*/LTR deletion by Southern blotting, suggesting that a considerable wild-type sequence was present (Fig. 3, panel M16b). Additionally, M16, but not other SIV_{sbbc}-inoculated animals, seroconverted to recombinant SIV_{mac239} Nef (supplied by AIDS reagent project, National Institute of Biological Standards and Control, Potters Bar, United Kingdom) by enzyme immunoassay (Fig. 4A).

To determine if the attenuated SIV infections initiated by

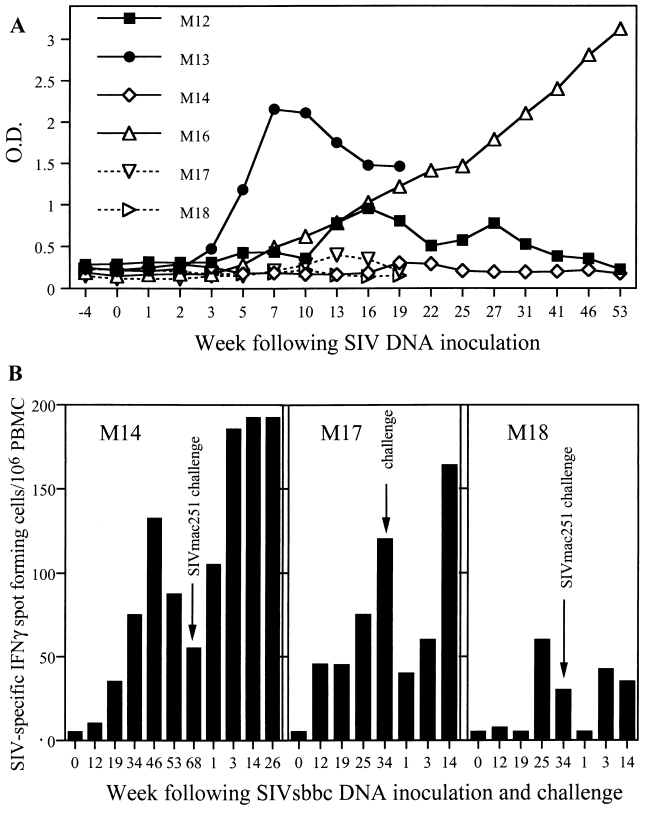

FIG. 4. (A) SIV Nef-specific antibodies following SIV DNA inoculation of macaques. Antibodies were detected against whole recombinant SIV Nef by enzyme immunoassay. Wild-type SIV_{mac239} DNA-inoculated animals M12 and M13 (closed symbols) had detectable anti-Nef antibodies, but only monkey M16 of the SIV_{sbbc}-inoculated animals developed anti-Nef antibodies. O.D., optical density. (B) ELISPOT detection of SIV-specific IFN-γ production by PBMC from SIV_{sbbc}-vaccinated macaques prior to, and following, SIV_{mac251} challenge.

proviral DNA inoculation could protect simians from a pathogenic challenge, the remaining three SIV_{sbbc}-inoculated animals and three naïve controls were challenged intrarectally with a stock of highly infectious SIV_{mac251} (20; R. Pal, submitted for publication). All control animals were shown to have seroconverted by particle agglutination assay and Western blotting (not shown). SIV was recovered by coculture from 10^4 or fewer of their PBMC (not shown), and their peak SIV RNA levels were high ($>10^7$ copies/ml) (Fig. 5A). One control animal (M20) developed diarrhea and weight loss and was euthanized 3 weeks after infection. The other two control animals (M19 and M22) had a progressive decline in $CD4^+$ T cells over a 32-week period (Fig. 5B).

All three macaques vaccinated with SIV_{sbbc} strains via DNA injection were protected from the high peak level of SIV RNA observed in the control animals 1 to 3 weeks following SIV_{mac251} challenge (Fig. 5A). However, progressively higher SIV RNA levels were detected in M18 over the first 11 weeks following SIV_{mac251} challenge and subsequently had a progressive decline in $CD4^+$ lymphocytes (Fig. 5). PCR of PBMC isolated from M18 following challenge demonstrated a wild-type-sized *nef*/LTR band that was shown to hybridize with an internal probe by Southern blotting and progressively became the dominant species (Fig. 3, panels M18). The two other SIV_{sbbc}-inoculated macaques, M14 and M17, were protected from SIV_{mac251} challenge, as they maintained low SIV RNA levels, stable $CD4^+$ lymphocyte levels, and a continued predominance of the PCR band of the *nef*/LTR deletion construct (Fig. 5 and 3, panels M14 and M17). Although protection was observed in two of three vaccinated animals, the small sample size meant that the result did not reach statistical significance (two sided, $P = 0.40$, Fisher's exact test).

To assess potential correlates of immunity, PBMC from vaccinated animals were assessed for the production of gamma interferon (IFN-γ) by ELISPOT (U-CyTech bv, Utrecht, The Netherlands) (2) in response to overnight stimulation with a 10-μg/ml concentration of whole Aldrithiol-2-inactivated SIV_{mne} or control microvesicles purified from the cell lines used to grow SIV_{mne} (kindly supplied by J. Lifson, National Cancer Institute, Frederick, Md. [15]). Antigen-stimulated PBMC (2×10^5/well) were incubated for 5 h in anti-IFN-γ monoclonal antibody-coated ELISPOT plates, and spots were detected using labeled anti-biotin antibodies. The number of spot-forming cells in control wells was always $\leq 20/10^6$ PBMC. Although the numbers of animals were small, PBMC from animals protected from SIV_{mac251} challenge (M14 and M17) had consistently high levels of production of IFN-γ in response to SIV antigen stimulation both prior to and following SIV_{mac251} challenge, which may have played a role in the

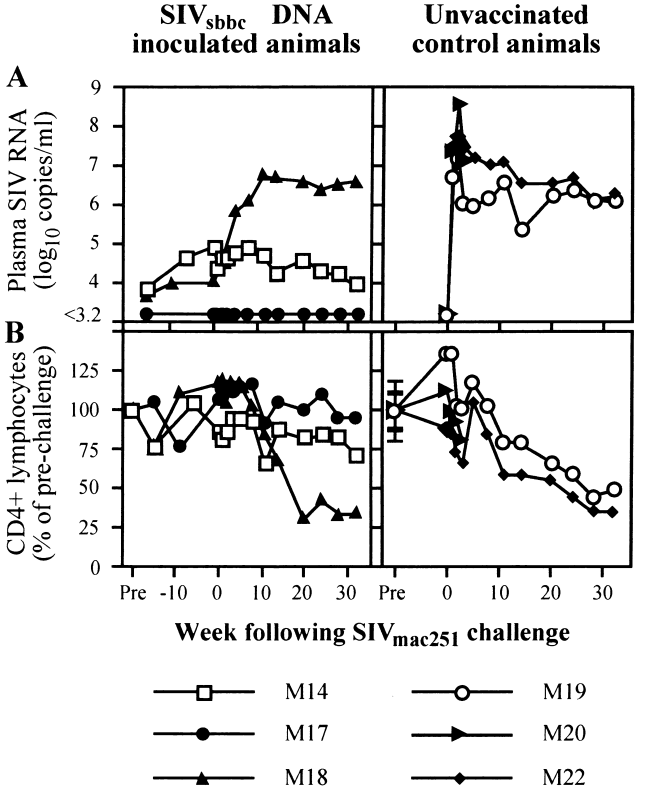

FIG. 5. Virologic and immunologic outcome of SIV_{mac251} challenge of SIV_{sbbc}-inoculated macaques and controls. (A) Plasma SIV RNA levels as measured by real-time RT-PCR. (B) $CD4^+$ lymphocyte numbers are percentages of prechallenge counts.

protective immunity observed (Fig. 4B). The rise in T-cell responses following challenge most likely reflects the nonsterilizing immunity and is consistent with the transient detection of wild-type-sized SIV DNA in M17 early on following challenge (Fig. 3), as observed in previous studies (10, 11).

In summary, we showed that attenuated SIV infections capable of stimulating SIV-specific T- and B-cell responses and protecting a proportion of monkeys against a virulent challenge can be initiated easily and reliably with inoculation of as little as 15 μg of proviral DNA. If live attenuated HIV vaccine strategies are ultimately proven to be safe (e.g., with the use of genes capable of turning replication on or off, as shown by Marzio et al. [17]), they could be delivered in the field using proviral DNA solutions rather than virus suspensions. Our studies also demonstrate that attenuating LTR mutations need to be engineered into both 5′ and 3′ proviral LTRs to prevent a rapid reversion to wild-type virus during in vivo replication, as detected in macaque M16. Although live attenuated lentiviral vaccines are currently insufficiently attenuated for use in humans (18), should current protein- and vector-based HIV-1 vaccines prove ineffective in clinical trials, our studies suggest practical and economical methods for developing and delivering attenuated lentivirus vaccines.

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