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DNA prime *Listeria* boost induces a cellular immune response to SIV antigens in the rhesus macaque model that is capable of limited suppression of SIV239 viral replication

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Abstract

DNA vaccines and recombinant *Listeria monocytogenes* that express and secrete SIV Gag and Env antigens were combined in a nonhuman primate prime-boost immunogenicity study followed by a challenge with SIV239. We report that recombinant DNA vaccine delivered intramuscularly, and recombinant *L. monocytogenes* delivered orally each individually have the ability to induce CD8$^+$ and CD4$^+$ T cell immune responses in a nonhuman primate. Four rhesus monkeys were immunized at weeks 0, 4, 8, and 12 with the pCSIVgag and pCSIVenv DNA plasmids and boosted with SIV expressing *L. monocytogenes* vaccines at weeks 16, 20, and 28. Four rhesus monkeys received only the *L. monocytogenes* vaccines at weeks 16, 20, and 28. A final group of monkeys served as a control group. Blood samples were taken before vaccination and 2 weeks post each injection and analyzed by ELISPOT for CD4$^+$ and CD8$^+$ T cell responses. Moderate vaccine induced SIV-specific cellular immune responses were observed following immunization with either DNA or *L. monocytogenes* vectors. However, the SIV antigen-specific immune responses were significantly increased when Rhesus macaques were primed with SIV DNA vaccines and boosted with the SIV expressing *L. monocytogenes* vectors. In addition, the combined vaccine was able to impact SIV239 viral replication following an intrarectal challenge. This study demonstrates for the first time that oral *L. monocytogenes* can induce a cellular immune response in a nonhuman primate and is able to enhance the efficacy of a DNA vaccine as well as provide modest protection against SIV239 challenge.
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Keywords: HIV/SIV vaccine; Prime/boost; *Listeria monocytogenes*; DNA vaccine

Introduction

The importance of a CD8$^+$ cytotoxic T lymphocyte response in controlling HIV replication is supported by a number of studies in humans and nonhuman primates (Amara et al., 2001; Barouch et al., 2001; Schmitz et al., 1999; Shiver, 2003; Shiver et al., 1995). The most efficient means of inducing a CD8$^+$ T cell mediated immune response is through live infection. As such, gene-delivered

Abbreviations: BHI, beef heart infusion; Lm, *Listeria monocytogenes*; Lm-SIV-Env-5′, recombinant *Listeria monocytogenes* strain that secretes the SIV239 Env polypeptide fragment residues 30 to 198; Lm-SIV-Env-3′, recombinant *Listeria monocytogenes* strain that secretes the SIV239 Env polypeptide fragment residues 298 to 525; Lm-SIV-Gag, recombinant *Listeria monocytogenes* strain that secretes SIV239 Gag; LP, lamina propria; MALT, mucosal associated lymphoid tissue; MLN, mesenteric lymph nodes; PBMC, peripheral blood mononuclear cells; PP, Peyer's patches.

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antigens are conceptually presented to the immune system in a manner similar to that which occurs during natural viral infection. The host-synthesized viral proteins are subjected to natural immune surveillance by the exogenous and endogenous antigen pathways resulting in both major histocompatibility complex (MHC) class I- and class II-driven cellular responses. Many of the HIV-1 vaccine approaches that are under development (Marsac et al., 2002; Rose et al., 2001; Seth et al., 1998, 2000; Vajdy et al., 2001; van Loon et al., 1996; Worku et al., 2001) deliver HIV-1 antigens to the host through recombinant viral infection. In addition, various combinations of the different strategies have demonstrated induction of cellular immune responses, which can impact viral replication in the SIV macaque model (Allen et al., 2000; Amara et al., 2001; Hanke et al., 1999). Our laboratory and others have demonstrated that DNA inoculation technology can be used to develop an HIV-1 antigen-specific immune response in mice, rhesus macaques, chimpanzees, and humans (Boyer et al., 2000; Kim et al., 2000; MacGregor et al., 2002; Vajdy et al., 2001; Wang et al., 1993, 1994, 1995). Furthermore, these vaccines induce a strong Th1-biased $CD8^+$ T cell-mediated immune response. However, these approaches have not, in general, used mucosal delivery schemes thought to be important for control of lentiviral replication. We reasoned that one useful approach would be to study the ability of a DNA vaccine combined with a mucosal delivery vaccine strategy to induce a cellular immune response.

Listeria monocytogenes, a gram-positive facultative intracellular bacterium, has been developed as a live vaccine vector (Weiskirch and Paterson, 1997). This vector induces weak humoral responses but a strong cellular response with both $CD8^+$ and $CD4^+$ T cell activation (Bishop and Hinrichs, 1987; De Libero and Kaufmann, 1986) of the Th1 phenotype (Hsieh et al., 1993a, 1993b; Mata and Paterson, 1999) in mice when delivered either intraperitoneally or orally. Earlier work described a genetic method to modify the chromosome of *L. monocytogenes* so that HIV gene products can be expressed as secreted proteins under the control of a copy of the strong promoter of the hemolysin (*hly*) gene, which encodes listeriolysin-O (Frankel et al., 1995). A *Listeria* vector expressing HIV-1 Gag (Lm-HIV-Gag) was shown to induce strong cell mediated immunity when delivered parenterally (Frankel et al., 1995; Mata and Paterson, 1999; Mata et al., 1998, 2001) or orally (Peters et al., 2003) and to protect against challenge with vaccinia virus expressing Gag in two strains of mice (Mata et al., 2001). However, *L. monocytogenes*-based vaccines for HIV or SIV antigens have not been examined previously in a nonhuman primate model.

Accordingly, we studied these two methods of vaccination, oral recombinant *Listeria monocytogenes*-based vaccines and intra-muscular DNA plasmid vectors, alone and in a prime-boost strategy. We focused on using two SIV antigens, Gag and Env. Three *L. monocytogenes* vaccines were constructed that expressed and secreted SIV Gag, a fragment of SIV Env that spanned residues 30 to 298 and a fragment of SIV Env that spanned 198 to 525. SIV-specific cellular immune responses were assessed by IFN-γ ELISpot in peripheral blood 2 weeks following each immunization with the DNA and *L. monocytogenes* vectors separately, and together, in Chinese Rhesus macaques. The immunization schedule is shown in Table 1. Although all the immunization strategies, we tested induced cell mediated immunity, as determined by ELIspot analysis, the SIV antigen-specific immune responses were significantly improved when the macaques were primed with SIV DNA vaccines and boosted with the SIV-expressing *L. monocytogenes* vectors delivered orally. To test whether levels of cellular immunity observed translated into protective levels of immunity, we challenged the animals intrarectally with a high challenge dose of SIV239mac 8 weeks following the last immunization. All monkeys did become infected following viral challenge. The monkeys that received DNA and were boosted with recombinant Listeria controlled viral load for a significantly longer period of time compared to the group that received recombinant Listeria and the control group. In addition, no significant latent virus levels were detected in resting $CD4^+$ T cells from the animals in the group that controlled viral load or in their inguinal lymph nodes.

Results

Chemistry and hematology safety studies following immunization with DNA and L. monocytogenes

The immunization schedule is shown in Table 1. Animals received four DNA vaccines followed by three *L. monocytogenes* boosts. All animals appeared in good health throughout the study as assessed by body weights and

Table 1
Immunization and challenge regimen

Group	Weeks 0, 4, 8, 12	Weeks 16, 20	Week 28	Week 36
1	DNA 2 mg SIVgag 2 mg SIVenv	*L. monocytogenes gag* and *env* vectors oral 3×10^{10}/dose	*L. monocytogenes gag* and *env* vectors oral 6×10^{10}/dose	2500 TCID50 SIV_{239}
2	–	*L. monocytogenes gag* and *env* vectors oral 3×10^{10}/dose	*L. monocytogenes gag* and *env* vectors oral 6×10^{10}/dose	2500 TCID50 SIV_{239}
Control	–	–	–	2500 TCID50 SIV_{239}

clinical parameters (listed in Table 2) measured in peripheral blood 2 weeks following each immunization. The animals did not suffer from any diarrhea or lethargy following immunization with *Listeria* and prior to challenge with SIV239. The liver can also be a site affected by systemic *Listeria* infection. Liver function is assessed by alkaline phosphatase and gamma glutamyl transaminase levels. The normal ranges for these two proteins are 120.5–1053 U/l and 24.4–108.9 U/l, respectively. In addition, a high mean corpuscular volume (MCV) is associated with liver disease, normal range is approximately 66–80 fl. All values remained within normal ranges. These results confirm there are no consistent pathological abnormalities observed as consequences of either SIV DNA vaccination, recombinant *L. monocytogenes* vaccination or a combination of the two vaccines in the prime/boost strategy.

Cellular immune response to SIV Gag and SIV Env following immunization with L. monocytogenes

We assessed the ability of recombinant *L. monocytogenes* vaccines expressing SIV antigens to induce a cellular immune response following oral inoculation of rhesus macaques. Animals were bled prior to, and 2 weeks following, each immunization (Table 1). PBMCs were isolated and incubated with SIV Gag peptide mix or SIV Env peptide mix overnight. The number of cells secreting IFN-γ in a Gag- or Env-specific manner was determined using a standard ELISpot assay. The results are presented in Figs. 1C and D, respectively. There were no significant responses observed following one immunization to either Gag or Env peptide pools. However, following two immunizations with recombinant *L. monocytogenes* 2 of 4 rhesus macaques responded to Gag. The mean number of Gag-specific IFN-γ secreting cells was 140 (±132) SFC per 1 million PBMCs which is statistically different to the unimmunized controls ($P < 0.05$). Furthermore, following 2 immunizations 2 of 4 rhesus macaques responded to Env. The mean number of Env-specific INF-γ secreting cells was 94 (±58) SFC per 1 million PBMCs ($P < 0.05$ compared to unimmunized control values). Finally, it appears that a third and final immunization with recombinant *L. monocytogenes* did not further enhance the vaccine-induced immune response to either antigen, suggesting an anti-Listeria response had developed that prevented vaccine boosting.

Table 2
Clinical tests performed to determine safety of DNA and *L. monocytogenes* immunization of rhesus macaques

Clinical chemistry values	Clinical hematology labs
Calcium	Platelets
Phosphate	White blood cells
Glucose	Hemoglobin
Blood urea nitrogen	Hematocrit
Creatine	Mean corpuscular volume
Protein	Mean corpuscular hemoglobin
Albumin	Mean corpuscular hemoglobin concentration
Globulin	Segmented neutrophils
Albumin/Globulin ratio	Band neutrophils
Total bilirubin	Absolute band count
Alanine aminotransferase	Monocytes
Alkaline phosphatase	Eosinophils
Aspartate aminotransferase	Basophils
Sodium	Atypical lymphocytes
Potassium	Total lymphocytes
Chloride	CD4
Cholesterol	CD8
Gamma glutamyl transaminase	CD4/CD8 ratio
	CD3/CD8 ratio
	CD3/CD4 ratio

Cellular immune response to SIV Gag and SIV Env following immunization with DNA vaccines

We also assessed the ability of rhesus macaques to respond to DNA vaccines expressing SIV Gag or SIV Env. The results are presented in Figs. 1E and F. Following one immunization, no antigen-specific immune responses were observed. However, following two immunizations 2 of 4 rhesus macaques developed an antigen response to Gag, mean 130 (±144) SFC per 1 million PBMCs. Following the third immunization 1 of 4 animals demonstrated an Env-specific immune response; mean 82 (±142) SFC per 1 million PBMCs. In addition, 2 of 4 animals demonstrated a positive response to SIV Gag, mean 180 (±182) SFC per 1 million PBMCs. However, animal 3232, which demonstrated an immune response following the second injection, did not demonstrate an immune response following the third or fourth immunization. Animal 3233 demonstrated a Gag-specific response following the third immunization, which was sustained following each subsequent immunization. Rhesus macaque 3239 which demonstrated an immune response to Gag following the second immunization had an immune response, which was sustained following each immunization. The mean number of INF-γ-producing cells following the fourth immunization in this nonhuman primate group was 153 (±123) SFC per 1 million PBMCs for Gag, and 50 (±78) SFC per 1 million PBMCs for Env. The response rates were, however, 2/4 for Gag and 1/4 for Env.

Cellular immune responses to DNA vaccines are boosted by recombinant L. monocytogenes-expressing SIV antigens

We next examined if *L. monocytogenes* would boost a DNA vaccine primed cellular immune response to the SIV antigens. As noted above, the mean number of INF-γ-producing cells following the final DNA immunization was 153 (±123) SFC per 1 million PBMCs for Gag, and 50 (±78) SFC per 1 million PBMCs for Env. These numbers were not significantly increased following the first boost with *L. monocytogenes*. However, following the second boost with *L. monocytogenes*, the response to Gag was 603 SFC per 1 million (±268), Fig. 2A ($P < 0.05$). Following

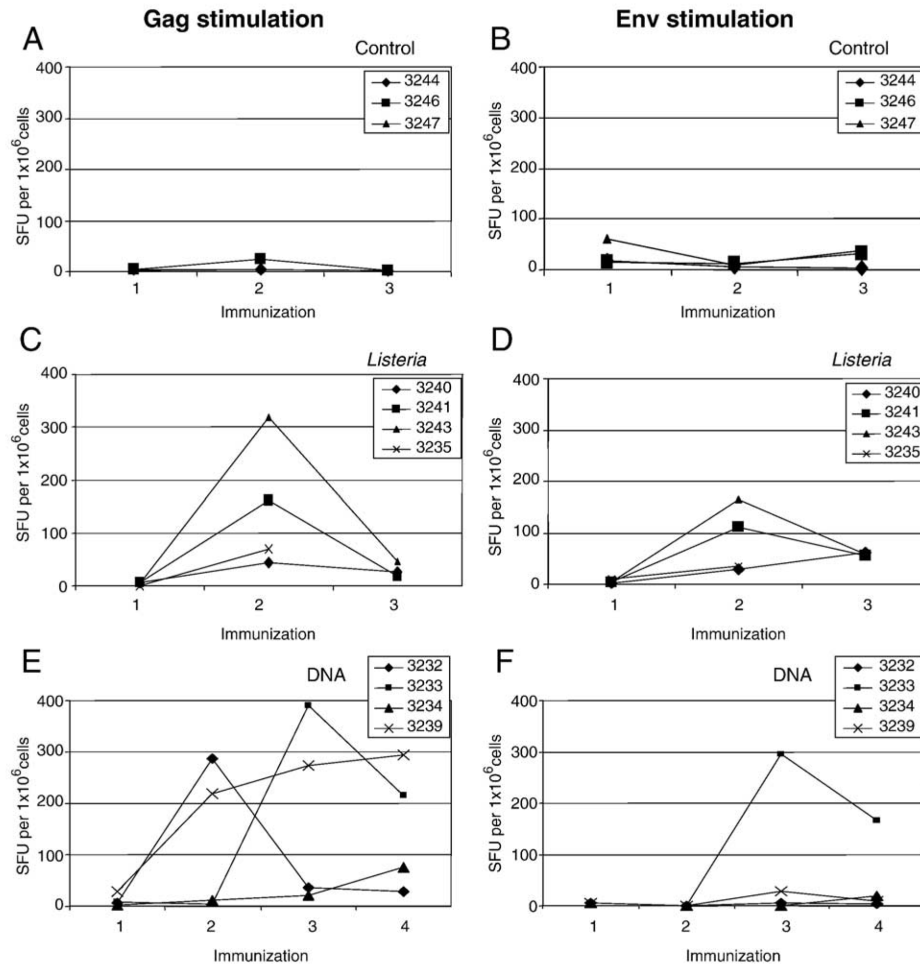

Fig. 1. IFN-γ-producing cells following immunization with either a *Listeria monocytogenes* vaccine or a DNA vaccine. Samples were taken before vaccination and 2 weeks post each injection. PBMCs were isolated by a standard ficoll-hypaque separation technique and assessed for a Gag antigen-specific response or an Env antigen-specific response by a standard ELISPOT. The top two panels show responses in control rhesus monkeys to (A) Gag and (B) Env. The middle two panels show responses in *L. monocytogenes* SIV immunized animals, after 1, 2, and 3 immunizations, to (C) Gag and (D) Env. The bottom two panels show responses in DNA immunized animals, after 1, 2, 3, and 4 immunizations, to (E) Gag and (F) Env.

the second boost with *L. monocytogenes*, the response to Env was 286 (±52), Fig. 2B ($P < 0.05$). In addition, 4 of 4 animals responded to both the Env and Gag antigens following the DNA prime and two boosts of recombinant *L. monocytogenes*. As observed earlier, a third and final boost with *L. monocytogenes* did not appear to enhance the immune response beyond what was seen following two immunizations with *L. monocytogenes*. This suggests that the *L. monocytogenes* vector induced an immune response against its own antigens that limited further boosting against the encoded SIV passenger antigens.

CD8$^+$ T cell immune responses to DNA vaccines are boosted by recombinant L. monocytogenes expressing SIV antigens

To assess if the cells secreting INF-γ were CD8$^+$ or CD4$^+$ lymphocytes we depleted PBMCs of CD8$^+$ T cells using magnetic beads coated with anti-CD8 monoclonal antibody (Dynal) and set up the remaining cells in a standard ELISpot assay. The immune response 2 weeks post the second *L. monocytogenes* boost is presented in Fig. 3 for individual macaques. It is clear that a CD8$^+$ SIV-specific immune response was induced to both the Env (Fig. 3C) as well as the Gag (Fig. 3D) antigens. The average Env-specific response prior to CD8$^+$ T cell depletion was 286 SFC/million PBMCs (±52 SFC/million PBMCs). However, following CD8$^+$ T cell depletion, the average Env-specific response was almost completely eliminated ($P = 0.0679$). The average CD8$^+$ T cell immune response to Gag prior to depletion was 603 SFC/million PBMCs (±268 SFC/million PBMCs), which decreased to 248 SFC/million PBMCs (±126 SFC/million PBMCs) following CD8$^+$ T cell depletion ($P = 0.0679$). This indicates a higher level of Gag antigen-specific CD4$^+$ T cell responses compared to the envelope responses. These trends were observed in all animals.

Viral loads

All animals were challenged with 2500 $TCID_{50}$ of SIV239 8 weeks after the last immunization. Data presented

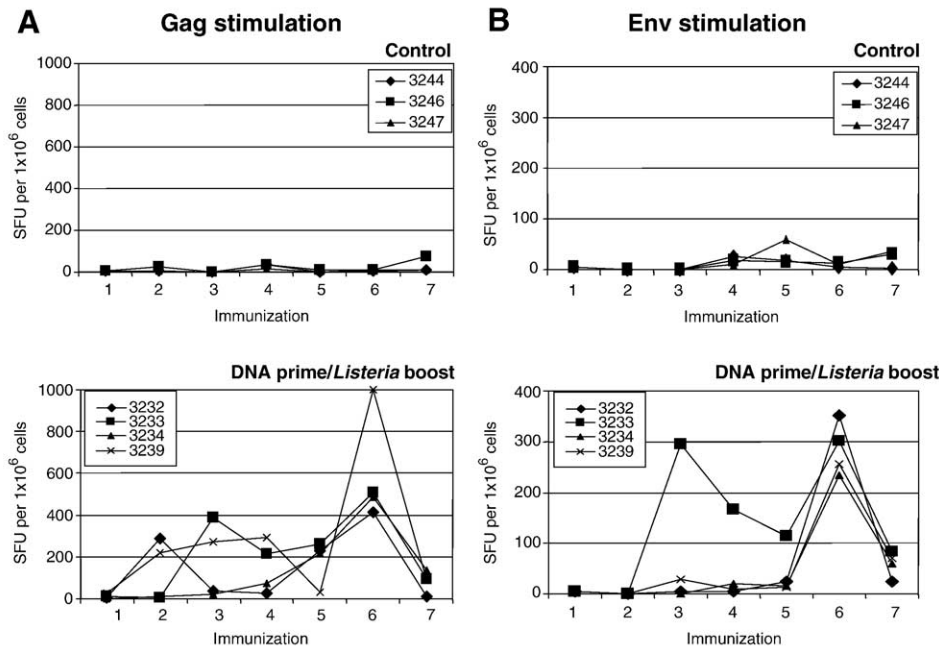

Fig. 2. SIV Gag- and Env-specific IFN-γ producing cells following DNA immunization and a boost with recombinant *L. monocytogenes* vaccine. PBMCs from control animals and animals immunized with pCSIVgag and pCSIVenv DNA plasmids followed by a boost with the *L. monocytogenes* vaccines were tested for their ability to respond in vitro to SIV Gag (A) or to SIV Env (B). PBMCs were isolated and were aliquoted at a concentration of 200,000 cells per well and mixed with SIV-specific peptides. The plates were incubated overnight and the number of IFN-γ-producing cells was enumerated.

in Fig. 4 shows that all animals were infected. However, the level of infection in animals 3240 (Fig. 4B) and 3239 (Fig. 4D) had low levels of infection with a peak viral load about 3 logs lower than any of the animals in the control group. There were no statistically significant differences between the control group (Fig. 4A) and the animals immunized with Listeria vector alone (Fig. 4B). Although one of three animals in the control group appeared to suppress viral load,

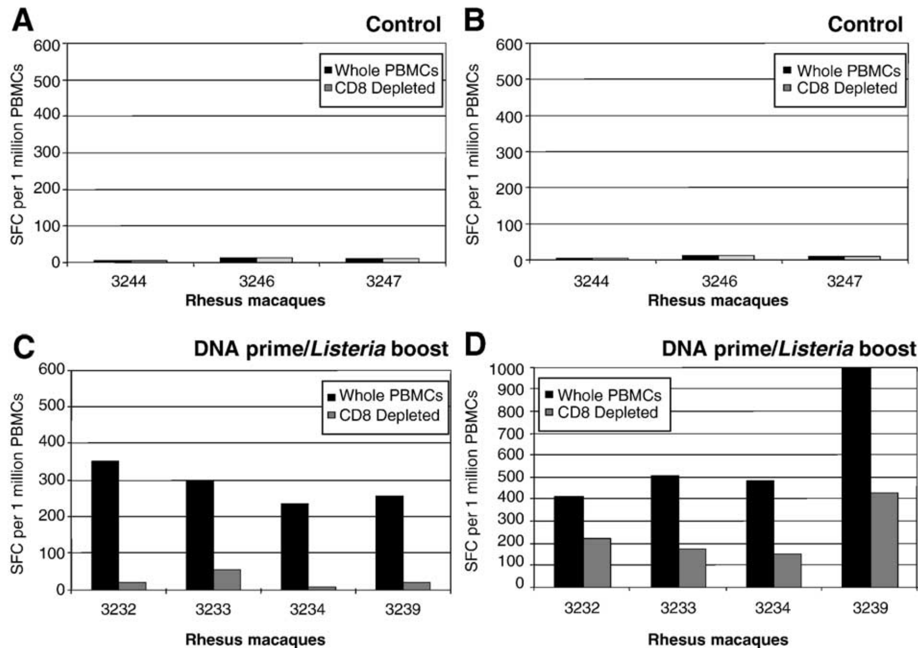

Fig. 3. $CD8^+$ IFN-γ-producing cells following DNA prime and *L. monocytogenes* vaccine boost. The $CD8^+$ antigen-specific immune response was assessed by a standard ELISPOT. PBMCs were isolated and depleted of $CD8^+$ cells and were aliquoted at a concentration of 200,000 cells per well and mixed with Env- or Gag-specific peptides. The plates were incubated overnight and the number of IFN-γ producing cells was enumerated. (A) PBMCs from control animals were assessed with Env peptides. (B) PBMCs from control animals were assessed with Gag peptides. (C) PBMCs from immunized animals were assessed with Env peptides. (D) PBMCs from immunized animals were assessed with Gag peptides.

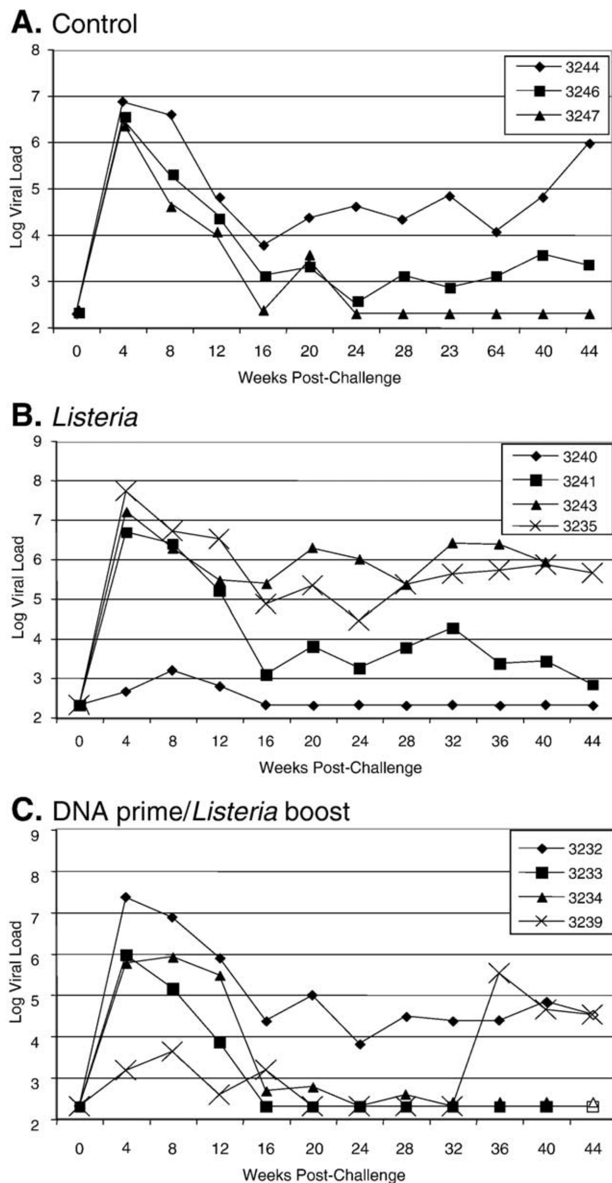

Fig. 4. Viral Loads following Challenge with SIV239. Samples were taken before and after SIV239 intrarectal challenge. PBMCs were isolated and viral loads were assessed by standard assays. (A) Control animals, (B) animals immunized with *L. monocytogenes* expressing Gag and Env DNA plasmids, and (C) animals immunized with pCSIVgag and pCSIVenv DNA plasmids followed by a boost with the *L. monocytogenes* vaccines.

this animal had a peak viral load that was comparable to all other animals in this study. In contrast, 3 out of 4 monkeys that received both DNA and Listeria controlled viral load in the blood. Following a peak viral load observed at 4 weeks post-challenge, viral loads decreased to set-point by week 16 post-challenge and remained there till week 32, post-challenge, at which time animal 3239 had viral breakthrough suggesting that there was viral escape. However, animals 3233 and 3234 maintained suppressed viral loads 44 weeks post-challenge. The group of animals that received a DNA prime followed by a Listeria boost were able to control viral loads for a significantly longer time period than the control group, (P = 0.0279 by Fischer's exact test).

Anti-SIV antibodies detection

We assessed sera for circulating anti-SIV antibodies using an anti-HIV-2 IgG ELISA Kit (Genetic Systems, Seattle, Wash.), which is cross-reactive for SIV antibodies. We did not detect any antibody responses as a result of the immunization regimens (data not shown). DNA immunization, *L. monocytogenes* or the two used in combination were unable to induce antibodies specific to SIV. This suggests that the immune responses generated by these vaccines were predominantly cell mediated. However, following challenge most animals rapidly demonstrated antibody responses that suggested that the immunized animals were primed for antibody development, Fig. 5. The animals that were immunized with recombinant Listeria alone clearly demonstrated a shorter time interval to the development of peak antibody levels. Three of four animals demonstrated a peak between weeks 6 and 10 after challenge which was significantly higher than control animals (P = 0.049). Animals 3240 did not demonstrate antibody at any time point; most likely, as Fig. 4B indicates, the level of infection in this animal was not sufficient to recall a humoral response and it was removed from this analysis. The control group of animals demonstrated peak antibody levels between weeks 10 and 20 in 3 of 3 animals challenged. The antibody responses in the DNA primed-Listeria boosted group had a response profile following challenge that varied between animals. Animals 3232 and 3233 had a continuing rise in antibody levels past 15 weeks. Animal 3234 reached a maximum response at approximately week 6, whereas animal 3239 had a low but detectable antibody response at the time of challenge, which was undetectable until approximately week 26.

Viral reservoirs

We assessed the viral reservoir status in all animals that demonstrated suppressed viral replication in two tissues. The frequency of resting CD4^{+} T cells, in peripheral blood, harboring replication competent SIV, was measured 25 weeks after challenge, Table 3. It was below the limit of detection in animals 3239 and 3240 and was barely detectable in animal 3233. Interestingly, these animals also had the lowest peak viral loads as well as suppression of viral replication. As we discussed above, 3240 did not become highly infected. Animal 3239 eventually had viral breakthrough demonstrating the persistence of SIV in a viral reservoir despite suppression of viremia to below detectable limits. Animal 3247, the macaque in the control group which was able to control viral load, demonstrated the highest level of infected resting CD4^{+} T cells, (Table 3).

We also examined inguinal lymph nodes, harvested from monkeys that appeared to control viremia: 3247 in the

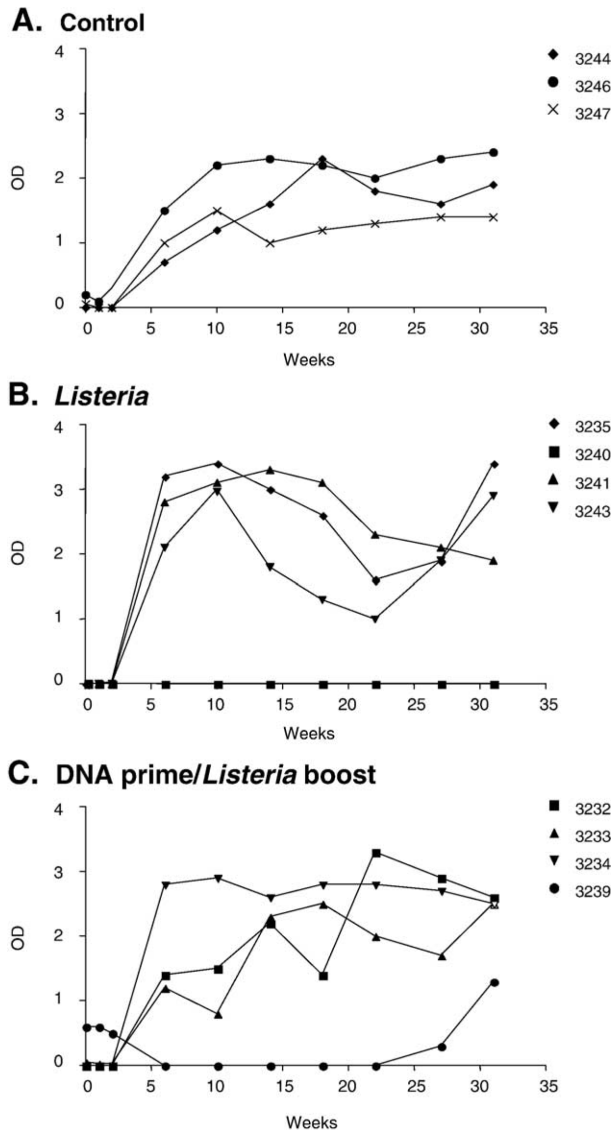

Fig. 5. Antibody to SIV Env. Sera was isolated at the time of challenge and following challenge and tested in a standard HIV-2 ELISA kit for binding to HIV-2 Env using OD units as a measure of antigen-specific responses. (A) Control animals, (B) animals immunized with *L. monocytogenes* expressing Gag and Env DNA plasmids, and (C) animals immunized with pCSIVgag and pCSIVenv DNA plasmids followed by a boost with the *L. monocytogenes* vaccines.

control group; 3233, 3239 in the DNA prime/Listeria boost vaccine group and 3240 in the group that received the Listeria vaccines alone. Lymph nodes were harvested at the time of sacrifice 44 weeks after viral challenge. Virus could only be detected in sections from monkey 3247 in the control group and 3239 where virus escape had occurred at about 32 weeks post-infection (Fig. 6).

Clinical outcome

Infected animals were monitored for clinical symptoms of disease and lymphocyte subsets. Euthanasia did not have to be performed on any animals in this study. Fig. 7 summarizes the $CD4^+$ lymphocyte level as number of cells per mm^3 of blood. All animals had a decrease in $CD4^+$ T cell numbers following infection. However, for those animals with the lowest viral set points, that is, 3233, 3234, and 3239 in the DNA-primed Listeria boosted group and 3235 and 3240 in the Listeria alone group, only 3234 failed to return to normal $CD4^+$ T cell levels. In addition, over time the DNA-primed Listeria boosted group did not demonstrate as significant a decrease compared to the control group (P = 0.0087).

Discussion

This is the first published study to examine the immunization potential of oral recombinant *L. monocytogenes* expressing SIV antigens in nonhuman primates. There are obvious advantages to immunizing with an oral vaccine. As the most widely used mucosal vaccination route, oral immunization has proven both practical and reliable in large-scale public health vaccination programs and generally elicits a high level of compliance from clinical cohorts enrolled in oral vaccine field trials (Sabin, 1985; van Loon et al., 1996). We found that Rhesus macaques immunized with *L. monocytogenes* by the oral route developed an antigen-specific cellular immune response to both SIVmac239 Env and SIVmac239 Gag following two immunizations with recombinant *L. monocytogenes*. There did not appear to be any boosting following a third immunization. It is possible that the immune response following this last immunization was limited to mucosal lymphoid sites and did not emerge in the periphery. However, more likely, the repeated immunizations with recombinant Lm-SIV vectors induced sufficient anti-vector immunity to interfere with the presentation of the transgene product upon subsequent boosts. We have recently shown that Lm-HIV-Gag is

Table 3
Viral reservoir in peripheral blood and resting $CD4^+$ T cells

Weeks post-challenge					
Macaque/ vaccine regimen	Peripheral blood[a]				Resting $CD4^+$ T cells[b]
	3 weeks	7 weeks	11 weeks	15 to 25 weeks	25 weeks
3233 DNA/Lm	143,980	<200	<200	<200	67
3234 DNA/Lm	820,860	480	600	<200	Not done
3239 DNA/Lm	4180	1500	<200	<200	<33
3240 Lm only	1520	<200	<200	<200	<33
3247 Control	49,560	220	2760	<200	1333

[a] SIV DNA copy eq/ml of plasma.
[b] SIV DNA copies per million resting $CD4^+$ T cells.

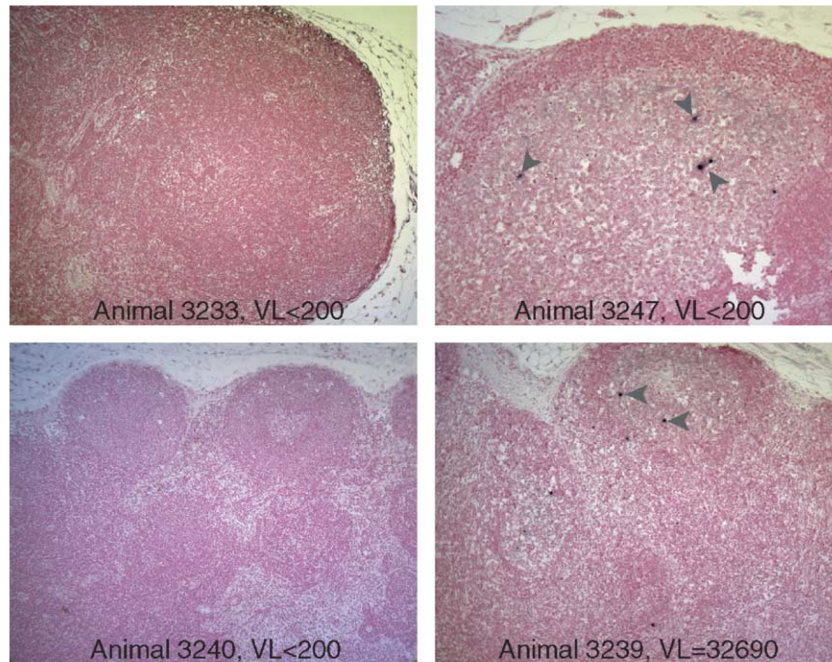

Fig. 6. Detection of SIV virus in inguinal lymph nodes by in situ hybridization. Lymph nodes were harvested at 44 weeks post-intrarectal challenge with SIV239. Multiple sections were cut and examined for the presence of virus (see arrows). Typical views are shown at 10-fold magnification for macaques 3233, 3240 and 3239. 3247 is shown at a magnification of 20-fold. The viral load (VL) in peripheral blood at the time of sacrifice is shown on the figure for comparison.

rapidly cleared from the periphery and MALT of mice upon a second oral immunization, although immune boosting was achieved (Peters et al., 2003). A lack of subsequent boosting after repeated immunization has been observed in other recombinant vaccine systems. In fact, a CTL response to SIV Gag was not further enhanced by a third immunization with NYVAC- (Hel et al., 2001) or modified vaccinia Ankara- (Hanke et al., 1999) based vaccine candidates.

Despite the immunogenicity of individual DNA or *Listeria* vaccine approaches and their ability to induce a cellular immune response, it was obvious that the HIV-1 antigen-specific immune response could be further enhanced and it was possible that a prime boost strategy would be a means of increasing the antigen-specific cellular immune responses. DNA prime followed by a boost with a recombinant live vector has the advantage of enhancing passenger antigen-specific responses while keeping the boosting of anti-vector responses to a minimum. Prime/boost vaccine regimens have been shown to be more effective than the individual vaccines for SIV for DNA based vaccines combined with modified vaccinia Ankara (Allen et al., 2000; Amara et al., 2001; Hanke et al., 1999), NYVAC (Hel et al., 2001), and Salmonella (Devico et al., 2002). In this paper, we show that DNA primed and oral *L. monocytogenes*-boosted animals developed higher immune responses than either vaccine used alone. Thus, 0.015% of total PBMCs were specific to SIVmac239 Gag antigen and 0.014% of total PBMCs were specific to SIVmac239 Gag antigen when rhesus macaques were immunized with either DNA or *L. monocytogenes* respectively. The immune responses, however, were increased 4-fold such that 0.06% of total PBMCs were specific to SIVmac239 Gag antigen in the animals primed with DNA and boosted with *L. monocytogenes*. Similarly, enhanced immune responses were observed to the Env antigen when nonhuman primates were primed with the DNA vaccine and boosted with the *L. monocytogenes* vaccines. Clearly, a prime boost strategy that combines these two vectors provides an immunological advantage over either approach when used alone.

It has long been thought (Mackaness, 1962) that humoral responses provide no resistance against listerial infections. Antibody titers either in peripheral blood or in gut associated lymphoid tissue are usually low in mice infected by *Listeria* either parenterally or orally (Pan et al., 1995). We also saw no increase in antibody titers against gp120 immediately after immunizing with *Listeria* either alone or in a prime/boost combination. It is surprising, therefore, that the animals that were immunized with *Listeria* alone clearly demonstrated an anamnestic antibody response to SIV Env, suggesting that priming by oral *Listeria* had taken place. Despite the more rapid and higher antibody titers obtained in this vaccine group, these did not appear to impact viral replication or protection of circulating $CD4^+$ T cell levels. Only one of the DNA-primed and *Listeria*-boosted vaccinated macaques demonstrated an anamnestic antibody response, macaque 3234. However, this group of macaques, primed with DNA before boosting with recombinant *Listeria* vaccine, had increased levels of SIV-specific cellular immune responses prior to challenge and were able

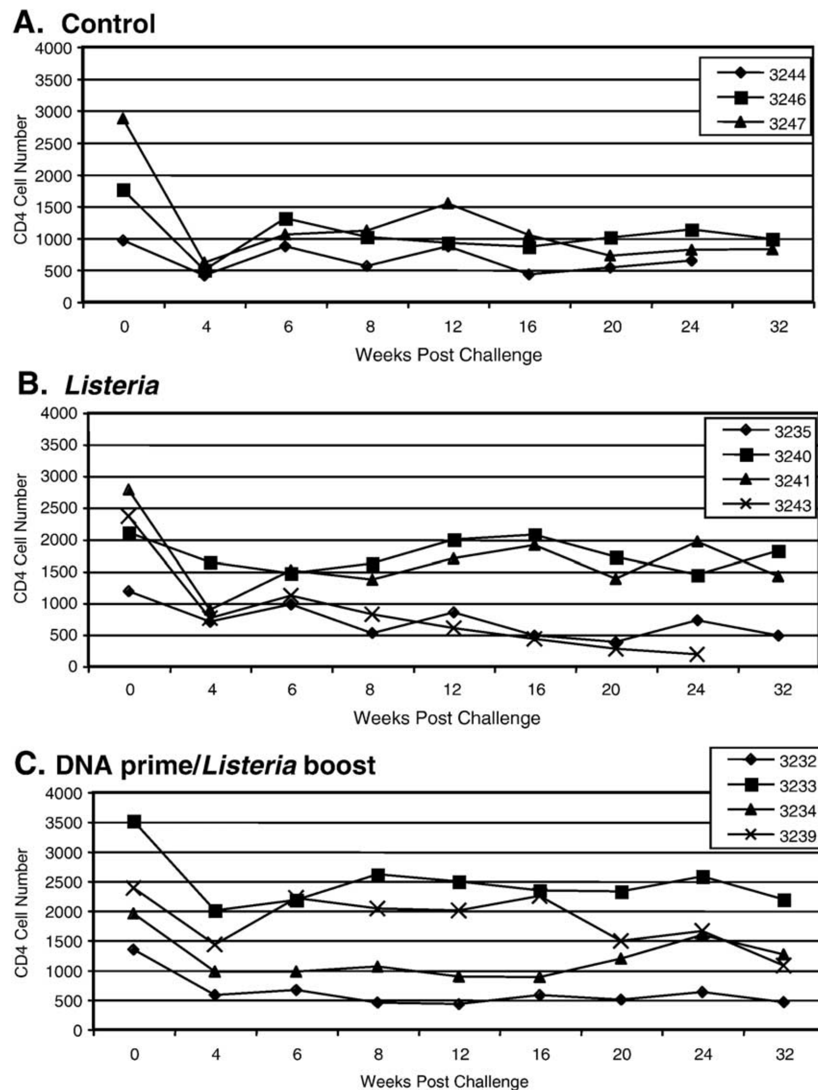

Fig. 7. $CD4^+$ Lymphocyte Counts following Challenge with SIV239. Samples were taken before and after SIV239 intrarectal challenge. PBMCs were isolated and lymphocyte counts were assessed by standard assays and expressed as cell number per mm^3. (A) Control animals, (B) animals immunized with *L. monocytogenes* expressing Gag and Env DNA plasmids, and (C) animals immunized with pCSIVgag and pCSIVenv DNA plasmids followed by a boost with the *L. monocytogenes* vaccines.

for a time to suppress viral replication and protect $CD4^+$ lymphocytes.

This conclusion is consistent with a large number of studies that have shown that the appearance of CTL in HIV infection coincides with a marked decrease in plasma viremia (Pantaleo et al., 1997) and there is a close inverse correlation between viral load and levels of circulating HIV-specific CTL (Ogg et al., 1998). The importance of HIV-specific CTL in HIV infection gains further support from the demonstration of HIV-specific CTL in highly exposed but apparently uninfected individuals (Kaul et al., 2000; Rowland-Jones et al., 1997). In this cohort study, an immune response was found systemically but was enhanced in the genital tract area at the likely site of virus exposure (Kaul et al., 2000). Clearly, in future studies, it will be important for us to measure the immune response to the DNA prime and *Listeria monocytogenes* vaccines in the mucosa. Yet under any circumstances, it is still not clear what the correlate of protection might be and whether we will be able to determine unambiguously what will lead to protection from SIV and subsequently HIV-1 infection or replication. Although, the level of effector cells as assessed by ELISpot may provide the best surrogate markers as to the level of antigen-specific lymphocytes that are available to sequester virus upon challenge.

We have not yet reached a level of protection that is satisfactory. There is an obvious need to induce a higher level of immune response that can suppress viral replication in all challenged animals. In addition, we may need to expand on the SIV genes included in our vaccines. Animal 3239 had a viral load that was suppressed below detectable limits for approximately 16 weeks. The breakthrough observed indicated that immune pressure did exist within this animal and that the virus was most likely able to escape

from the immune response. Studies completed with single antigens demonstrate a swift immune escape phenomenon (Vogel et al., 2003). A vaccine-elicited, single viral epitope-specific cytotoxic T lymphocyte response does not protect against intravenous, cell-free simian immunodeficiency virus challenge, (Yasutomi et al., 1995). However, in this paper, we have demonstrated the promise of these two vaccines, as a combined strategy for an HIV vaccine and their clear ability to induce an immune response that will impact on mucosal challenge.

Material and methods

Animals

Male and female Chinese rhesus macaques (Macaca mulatta) 3 to 8 years old were evaluated at the start of the study. The rhesus macaques were housed at Southern Research Institute in Frederick, MD under USDA approved conditions. These facilities are accredited by the American Association for the Accreditation of Laboratory Animal Care International and meet National Institute of Health standards as set forth in the Guidelines for Care and Use of Laboratory Animals.

Engineering and preparation of DNA plasmids

The pCSIVenv and the pCSIVgag plasmids expressing the SIV envelope and core protein, Gag, are Rev-independent expression vectors that have been codon optimized for high level expression as previously described (Nappi et al., 2001). Codon optimization mutates the DNA sequence without disrupting the amino acid sequence. Ultimately, there is mutation or elimination of the RRE inhibitory sequence, which leads to the more abundant translation of tRNA. Plasmids were purified by Puresyn (Malvern, PA). DNA was formulated in 0.15 M citrate buffer and 0.25% bupivicaine at a pH of 6.5.

Recombinant Listeria monocytogenes constructs

The recombinant *Listeria monocytogenes* vaccines used in this study are based on strain 10403S serotype 1 and carry a single copy of each SIV strain 239 antigen gene stably integrated into the listerial chromosome using a previously described method of homologous recombination (Mata et al., 2001). Lm-SIV-antigen strains were generated by introducing the SIV genes directly into the *L. monocytogenes* chromosome under the control of the *hly* promoter and including the *hly* signal sequence using plasmid pZY-55, which is a modified shuttle vector pKSV7. The pKSV7 was modified by replacing its polylinker region with an expression cassette containing a 1.6 KB *Eco*RI–*Hin*dIII fragment containing a portion of the orfXYZ region of the *L. monocytogenes* chromosome to provide a homologous region for subsequent allelic exchange of the plasmid into the chromosome and insertion of the genes into the orfZ region of the listerial chromosome. To drive the expression and secretion of the SIV gene products, a DNA fragment containing a copy of the 288-nucleotide promoter sequence of the *L. monocytogenes* hemolysin gene, signal sequence and N-terminal cleavage site was introduced into a *Kpn*I site in the orf Z region. PCR primers were 5′-GGGGTACCCTCCTTTGATTAGTATAT-3′ (engineered *Kpn*I site underlined) and 5′-CCATACTATATGTTTTAATTTATTGAATGCAGATGCATC-3′. The orfXYZ fragment was further modified by SOEing (gene Splicing by Overlap Extension) PCR to include a multicloning site for the insertion of foreign genes and a flag tag (GATTACAAGGATGACGACGATAAG) that encodes the sequence DYKDDDDK, to facilitate the detection of the gene product by Western blot.

To generate Lm-SIV-Gag the SIV *gag* gene was excised from pCR2.1 by double digest with Bgl I and Nhe I and ligated into pZY-55. The SIV *gag* gene was amplified by PCR using the primers 5′-GGAGATCTTACATGTTGAAGCATGTAGTA-3′ (engineered *Bgl*II site underlined) and 5′-GGGCTAGCCTGGTCTCCTCCAAAGAGA-3′ (engineered *Nhe*I site underlined) and ligated into the *Bam*HI and *Xba*I cloning sites in the *orfXYZ* homology region of pZY-55 downstream from the *hly* promoter and signal sequence. *L. monocytogenes* strain 10403S was transformed with pZY-55-SIV Gag. Clones were screened for integration of the SIV *gag* gene cassette into the *orfZ* domain. The Gag protein is expressed and secreted by Lm-SIV-Gag as determined by Western blot with an anti-flag antibody.

Because of difficulties in expressing SIV *env* as a secreted product, two SIV Env constructs were made that together overlap the entire gp120 sequence. Lm-SIV-Env-5′ expresses the fragment residues 30 to 298 (omitting the signal sequence) and includes domains I, II, and III of SIV Env. This fragment was amplified from the *env* gene using primers 5′-GGTCTAGATTAAGTCCTATTATCCCTACC-3′ (with engineered *Xba*I site underlined) and 5′-GGGGATCCGGTGTACCAGCTTGGAGG-3′ (*Bam*HI site underlined) and ligated into the *Bam*HI and *Xba*I cloning sites in the orfXYZ homology region of pZY-55 downstream from the *hly* promoter and signal sequence. In a similar manner Lm-SIV-3′ was constructed that represents residues 198 to 525 and encompasses domains III, IV and V of the SIV gp120 polypeptide chain. The fragment was amplified from the *env* gene using primers 5′-GGGGATCCTATTGGGATGCTATTAGA-3′ (with engineered *Bam*HI site underlined) and 5′-GGTCTAGATCTTTTATTTCTTGAGGTGCC-3′ (with engineered *Xba*I site underlined). The secretion of polypeptide fragments of an appropriate size was verified by Western blot.

Prior to their use, all strains of bacteria were mouse-passaged in order to stabilize their behavior in vivo (28). The bacteria were propagated on BHI (Difco, Detroit, MI). Bacterial aliquots were stored at −70 °C. An inoculum of

bacteria was prepared for oral immunization by thawing an aliquot and appropriately diluting it in sterile PBS.

Immunization schedule and sample collection

Three groups of Chinese rhesus macaques were immunized as outlined in Table 1. Four rhesus macaques were immunized at weeks 0, 4, 8, and 12 with 2 mg each of the pCSIVgag and pCSIVenv DNA plasmids. The DNA immunizations were followed by three boosts with recombinant *L. monocytogenes* at weeks 16, 20, and 28. The *L. monocytogenes* was delivered orally as an equal mixture of 10^{10} Lm-SIV-Gag and Lm-SIV-Env-5′ and -3′ providing a total dose of 3×10^{10} bacteria for the first two immunizations and 6×10^{10} bacteria/dose for the final immunization. A second group of four rhesus macaques received the *L. monocytogenes* delivered orally as 3×10^{10} bacteria/dose for two immunizations and 6×10^{10} bacteria/dose for the third immunization. Three rhesus macaques remained naive as controls. Heparinized blood was obtained for isolation of PBMCs and whole blood was taken for sera samples. Samples were collected prior to vaccination and 2 weeks after each injection. PBMCs were isolated by a standard ficoll-hypaque separation technique.

Peptides

One hundred and twenty five peptides corresponding to the entire coding region SIVmac239 Gag protein and 212 peptides that encompassed SIVmac239 Env protein were obtained from the AIDS Reagent Reference Repository (NIH). These 15-mers overlapping by 11 amino acids were resuspended in DMSO at a final concentration of 100 μg/ml and equal amounts of each peptide were mixed as one single pool for Env and one for Gag for ELISpot analysis.

ELISpot assay

ELISpot was performed using IFN-γ reagents from MabTech (Sweden) and nitrocellulose plates, (Cellular Technologies or Millipore). Briefly, plates were coated with 7.5 μg/mL IFN-γ capture antibody and incubated overnight at 4 °C or at room temperature for 2 h. After washing with sterile PBS, the peptide pools (100 μg/ml) were diluted 1:200 in culture medium (RPMI 1640 containing 2 mM L-glutamine, 100 IU/ml penicillin and 100 IU/ml streptomycin) and 100 μl was transferred into 6 wells. The plates were sterilized by UV light for 15 min. Cells in culture media were added to the wells in triplicate at a concentration of 200,000 cells per well. A second set of PBMCs was depleted with anti-human CD8 depletion beads according to manufacturer's protocol (Dynal) before plating in triplicate. The plate was incubated for 24 h at 37 °C, then washed with PBS. Biotinylated antibody at a concentration of 1 μg/ml was added and incubated overnight at 4 °C. After washing streptavidin-AP was added at a concentration of 1 ng/ml and incubated for 2 h at room temperature. Plates were washed with PBS. BCIP/NBT was added and incubated in the dark until spots appeared. The reaction was stopped by rinsing several times with tap water. After drying, the plates were scanned and counted using a Cellular Technologies ELISpot reader. A positive response is defined as 50 spot forming units (SFU) per million PBMCs. The background observed in this study was below 20 SFU per million PBMCs.

Anti-SIV antibody detection

Circulating anti-SIV antibodies were detected using an HIV-2 ELISA (Genetic Systems, Seattle, Wash.) that is cross-reactive for SIV antibodies. The ELISA used a fixed plasma dilution of 1:200 and was run as directed using a Vmax kinetic ELISA reader (Molecular Devices, Menlo Park, CA). Values of optical density (OD) units minus background were used as ELISA units.

Viral challenge

All animals were challenged intrarectally with 2500 $TCID_{50}$ of SIV239 8 weeks after the last immunization.

Flow cytometry

Peripheral blood lymphocyte subset analysis was performed on a FACS-scan flow cytometer (Becton-Dickinson, Mountain View, CA) using a panel of mouse anti-human monoclonal antibodies known to cross-react with macaque receptors. The lymphocyte subsets were stained with CD-2 (T cells) (clone 39C1.5 FITC, Coulter, Miami, FL), CD-20 (Leu-16-PE, B cells) (Becton-Dickinson), CD-3 (SP34-PE, Pharmagen, San Diego)/CD-4 (Leu-3a-FITC, Becton-Dickinson) and CD-3/CD-8 (Leu-2a-PerCP, Becton-Dickinson). CD4 cells as cell number per mm^3 are reported. Staining was performed on whole blood preparations. Analysis was performed on lysed and paraformaldehyde-fixed cells.

SIV viral RNA quantitation

SIV viral RNA was quantitated in peripheral blood using a previously described procedure (Suryanarayana et al., 1998). 500 μl of plasma was added to 1 ml of DPBS and spun for 1 h at 10,000 RPM. The viral pellet was then lysed using RNASTAT-60 (Tel-Test "B"). The samples were then amplified as previously described (Suryanarayana et al., 1998), with the exception of the primers and probe. The primers and probe used were SIV-F 5′AGTATGGGCAGCA-AATGAAT 3′, SIV-R 5′TTCTCTTCTGCGTGAATGC 3′, and the probe SIV-P 6FAMAGAT-TTGGATTAGCA-GAAAGCCTGTTGGA-TAMRA. This assay has a nominal

threshold sensitivity of 200 copy eq/ml of plasma (Suryanarayana et al., 1998).

Latent viral reservoirs

PBMC were isolated from heparinized blood using a Percoll gradient centrifugation procedure. The resulting PBMC were depleted of adherent cells and then depleted of unwanted cell populations using monoclonal antibodies and magnetic beads conjugated with anti-mouse IgG. Therefore, our procedure used the following antibodies to deplete unwanted cell populations: B cells, anti-CD20, NK cells, anti-CD16, $CD8^+$ T cells, anti-CD8, monocytes, anti-CD14, activated $CD4^+$ T cells, anti-CD25, anti-CD69, and anti-HLA-DR. By staining cells with a cocktail of antibodies to these markers followed by depletion with anti-IgG-conjugated magnetic beads, we obtain populations of $CD4^+$ $HLA\text{-}DR^-$ cells that are 80% pure. A subsequent sorting step gives us >98% purity. Virus released from latently infected cells was amplified by coculture of resting $CD4^+$ T cells with CEMx174 cells which are permissive for high level replication of most SIV isolates including the SIV239 strain were used. After 2–3 weeks of coculture, supernatants were tested for the presence of SIV p27 antigen by ELISA. Quantitation of latently infected cells is achieved by setting the assay up in a limiting dilution format. In addition to virus culture approaches, molecular approaches for the detection of cells in the post-integration stage of latency were used. Primer hybridizing with conserved regions of Alu sequences were used together with an outward directed primer in the SIV LTR to detect integrated SIV provirus in an unambiguous way. This produced a band of a single size from diverse integration sites. In each reaction, it was critical to include a control in which the initial Alu–PCR is run for zero cycles. This assured the bands obtained in the nested reaction were dependent upon the initial integration-dependent Alu–PCR reaction. Detection was dependent upon the initial Alu–PCR reaction.

Detection of virus in inguinal lymph nodes by in situ hybridization

Inguinal lymph nodes were harvested from macaques after sacrifice 44 weeks following challenge with SIV239. Formalin-fixed, paraffin-embedded tissues were sectioned (4 to 5 mm thickness) and placed on DEPC water-N-tris(hydromethyl) methyl-2-aminoethanesulfonic acid (TES)-coated glass slides before being dried at room temperature overnight. The slides were then heated for 5 min and were then washed sequentially (twice for 5 min) in a mixture of xylene, 100% ethanol, 95% ethanol, and DEPC-treated water. The slides were then incubated with 5 mM levamisole for 20 min, 13 SSC (13 SSC is 0.15 MNaCl plus 0.015 M sodium citrate) for 2 min, and a solution containing 25 mg of proteinase K per ml in 10 mM Tris (pH 7.4) and 0.2 mM CaCl2 at 37 °C for 10 min before further incubations in 0.1 M glycine in PBS for 5 min, 13 PBS for 5 min, and 0.1 M triethanolamine − 0.25% acetic anhydride for 10 min. Slides were prehybridized in prehybridization-hybridization buffer (50% formamide, 43 SSC, 13 Denhardt's solution, 4 mM $NaPO_4$, 0.1% SDS, 5% dextran sulfate, 250 mg of tRNA per ml, 250 mg of salmon sperm DNA per ml in DEPC water) in a preheated humidity chamber under a coverslip for 15 min. The probe was added to the hybridization buffer to a final concentration of 2.5 ng/ml, and the slides were placed under coverslips, sealed with rubber cement, heated to 65 °C for 5 min, chilled on ice, and incubated overnight at 50 °C. Following hybridization, the coverslips were removed and the sections were washed in 23 SSC −50% formamide solution at 50 °C for 5 min and then for 1 h, twice for 1 min in 23 SSC, and for 30 min in an RNase solution at 37 °C (RNase T1 and RNase A in 23 SSC). These washing steps were repeated, and the slides were blocked with a buffer containing 2% horse serum, 0.3% Tween 20, 150 mM NaCl, and 100 mM Tris (pH 7.4) for 1 h. Following the blocking, the slides were incubated for 1 h with sheep antidigoxigenin alkaline phosphatase conjugate at a 1:5,000 dilution in PBS − 2% horse serum, were washed twice, and were incubated with nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate toluidinium (NBT-BCIP) substrate in the dark at room temperature for 2 h. The slides were counterstained with nuclear fast red, dehydrated, and placed under coverslips.

Statistical analyses

Nonparametric statistics (Wilcoxon rank-sum test and Friedman two way analysis of variance) were used to compare antibody titers, $CD4^+$ T cell counts and ELISPOT data among control and vaccinated groups at different time points. ELISPOT means before and after CD8 depletion were examined for statistical differences using the Wilcoxon signed-rank test for paired data. Comparisons among proportions were done using Fisher exact test.

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