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Protection from Immunodeficiency Virus Challenges in Rhesus Macaques by Multicomponent DNA Immunization

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Multicomponent DNA vaccines were used to elicit immune responses, which can impact viral challenge in three separate rhesus macaque models. Eight rhesus macaques were immunized with DNA vaccines for HIV env/rev and SIV gag/pol and were challenged intravenously with 10 animal infective doses (AID_{50}) of cell-free SHIV IIIB. Three of eight immunized rhesus macaques were protected, exhibiting no detectable virus. Animals protected from nonpathogenic SHIVIIIB challenge were rested for extended periods of time and were rechallenged first with pathogenic SIV_{mac239} and subsequently with pathogenic SHIV89.6P viruses. Following the pathogenic challenges, all three vaccinated animals were negative for viral coculture and antigenemia and were negative by PCR. In contrast, the control animals exhibited antigenemia by 2 weeks postchallenge and exhibited greater than 10 logs of virus/10^6 cells in limiting dilution coculture. The control animals exhibited CD4 cell loss and developed SIV-related wasting with high viral burden and subsequently failed to thrive. Vaccinated animals remained virus-negative and were protected from the viral load, CD4 loss, disease, and death. We observed strong Th1-type cellular immune responses in the protected macaques throughout the study, suggesting their important roles in protection. These studies support the finding that multicomponent DNA vaccines can directly impact viral replication and disease in a highly pathogenic challenge system, thus potentially broadening our strategies against HIV.

INTRODUCTION

Since its initial development in the early 1990s, DNA vaccine strategy has become one of the key technologies in vaccine development. Countless academic and government laboratories have DNA vaccine programs, which target diseases ranging from TB to HIV as well as for the immunotherapy of various types of cancer (Donnelly *et al.,* 1997; Weiner and Kennedy, 1999). In addition, several biotechnology companies as well as some large pharmaceutical companies have active DNA vaccine programs. Even though the early clinical results show the glimpse of potential utility of this strategy, further testing in animal models and humans is important for the development of clinically relevant vaccines (Boyer *et al.,* 2000; Wang *et al.,* 1998).

Primates represent relevant models for HIV vaccine evaluation (Hulskotte *et al.,* 1998; Marx *et al.,* 1993). For HIV vaccine studies, these primate challenge models can be segregated into three categories: the HIV challenge model in chimpanzees and the SIV and chimeric SIV/HIV-1 (SHIV-1) challenge models in macaques (Luciw *et al.,* 1995; Ranjbar *et al.,* 1997). Chimpanzees can be infected by HIV isolates from humans; however, they rarely develop disease and are limited in quantity—a problem for vaccine testing. In contrast, the SIV challenge model uses an SIV virus, which replicates to high levels in rhesus macaques and causes a HIV-like disease in this plentiful species. In an effort to test HIV envelope immunogens in an animal model, the SHIV viruses were constructed by replacing SIV envelope genes with specific HIV-1 envelope genes (Schultz and Hu, 1993). SHIV viruses replicate in macaques and represent an infectious challenge model for HIV-1 envelope-based vaccines. Furthermore, certain SHIV strains such as SHIV 89.6P are pathogenic.

In this study, we examined the effect of multicomponent DNA vaccine immunization on viral challenges in rhesus macaques. The animals were immunized with a multicomponent DNA vaccine cocktail consisting of DNA plasmids expressing HIV env_{MN} and SIV gag/pol genes as well as those expressing cytokine genes. We observed that three of eight rhesus macaques immunized with multicomponent DNA vaccines were protected from a subsequent intravenous (iv) challenge with SHIVIIIB virus and exhibited no detectable virus in the limiting dilution coculture assay. Animals protected from nonpathogenic SHIVIIIB challenge were rested for long pe-

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riods of time and were rechallenged with pathogenic SIV_{mac239} and SHIV89.6P viruses. Interestingly, all three rhesus macaques were protected from the viral load, CD4 loss, disease, and death in these multiple pathogenic challenge models. These results suggest potential utility of multicomponent DNA vaccine strategy.

RESULTS

Analysis of DNA immunization in a SHIV IIIB challenge

Eight rhesus macaques were immunized with multicomponent DNA vaccines and two rhesus macaques were immunized with control plasmids as described under Materials and Methods. The potential effects of cytokine coimmunization on DNA vaccine-induced immune responses in rhesus macaques have been previously published (Kim *et al.,* 2000, 2001). Due to the small number of animals for each cytokine group, all DNA vaccine-immunized rhesus macaques (eight total) were pooled as one group prior to the SHIVIIIB challenge.

At week 53 (4 weeks after the last immunization), eight rhesus macaques immunized with multicomponent DNA vaccine cocktail as well as two control macaques were challenged by iv route with 10 animal infective doses (AID_{50}) of cell-free SHIV IIIB. Animals were then bled at 2, 3, and 4 weeks after the challenge and were assessed for protection from infection using a standard sensitive limiting dilution coculture analysis (Wyand *et al.,* 1996). As expected, both control macaques were infected within 2 weeks of viral challenge (Fig. 1). In contrast, we observed that three of eight immunized animals were able to prevent viral antigenemia due to iv challenge and exhibited no detectable virus in the limiting dilution coculture assay.

Based on the humoral immune responses measured in the protected and unprotected macaques, neutralizing antibody responses do not appear to correlate with control of viral replication and infection in this study (Kim *et al.,* 2000). Comparison of cell-mediated immune responses (CMI) in protected and nonprotected rhesus macaques suggests the importance of CMI for protection (Table 1). Based on these results, protection seemed to favor stronger CTL and/or stronger and more frequent LPAs, particularly to gag. To further characterize the cellular immune responses, we also analyzed the cytokine expression profiles from individual animal's stimulated T cells immediately prior to challenge at week 53. Cytokines play a key role in directing and targeting immune cells during the development of the immune response. For instance, IFN-γ is produced by Th1-type $CD4^+$ and $CD8^+$ T cells and is intricately involved in the regulation or development of antiviral T-cell-mediated immune responses (Clerici and Shearer, 1993; Rosenberg *et al.,* 1997). In contrast, IL-10 is produced by many cell types, including putative Th2 lymphocytes, and has been shown to be a potent Th2-type cytokine (Fiorentino *et al.,* 1989). Thus, analysis of these cytokines secreted by stimulated T cells may be important in elucidating the extent of cell-mediated responses following immunization (Lekutis *et al.,* 1997). As shown in Table 2, the stimulated T cells from the protected rhesus macaques produced significantly higher levels of IFN-γ than the unprotected animals (both control and immunized). On the other hand, the levels of IL-10 produced by the protected and the unprotected groups were similar.

Accordingly, several reports illustrate that chemokines can also modulate immune responses or can have a role in viral infection (Heeny *et al.,* 1997; Lehner *et al.,* 1996). We examined the expression profiles of β-chemokines (MIP-1α, MIP-1β, RANTES, and MCP-1) from immunized animals at week 53, just prior to challenge. The β-chemokines MIP-1α, MIP-1β, and RANTES are the major HIV suppressive factors produced by $CD8^+$ T cells for macrophage-tropic, but not T-cell-tropic, viruses (Cocchi *et al.,* 1995; Doranz *et al.,* 1996; Dragic *et al.,* 1996). Although MCP-1 has not been identified as one of the HIV suppressive factors, it has been shown to play a role in cellular immune expansion in the periphery (Kim *et al.,* 1998a). The stimulated T cells from the protected rhesus macaques produced significantly higher levels of MIP-1β and RANTES than the unprotected animals (with P values of 0.05 and 0.02, respectively) (Table 2). In contrast, the levels of MIP-1α and MCP-1 production by the two groups were not significantly different. Therefore, by four criteria—LPA responses, CTL responses at time of challenge, expression of IFN-γ, and expression of chemokines, RANTES and MIP-1β—a cellular bias appears to favor protection in this SHIV IIIB challenge study.

Protection from a pathogenic SIV_{mac239} challenge

SIV challenge models in macaques represent relevant model systems for studying the immunology and disease pathogenesis from immunodeficiency virus. In addition to the test against infectious virus afforded by the SHIV IIIB challenge, pathogenic SIV challenge allows the evaluation of vaccination strategy against disease pathogenesis. It has been, in general, more difficult to protect vaccinated primates against pathogenic SIV challenge models.

A study by Robinson *et al.* demonstrated that a prime boost approach supported the finding that intradermal DNA vaccination followed by pox viral booster could control pathogenic infection in most vaccinated animals (Robinson *et al.,* 1999). We next sought to confirm and extend this finding in this DNA-based study. The three protected monkeys from SHIV IIIB challenge were rested for 6 months. Then, these animals were boosted with 1 mg of SIV env and gag/pol DNA constructs at weeks 81 and 85. Subsequently, we examined the level of antigen-specific lymphoproliferative responses against SIV p27

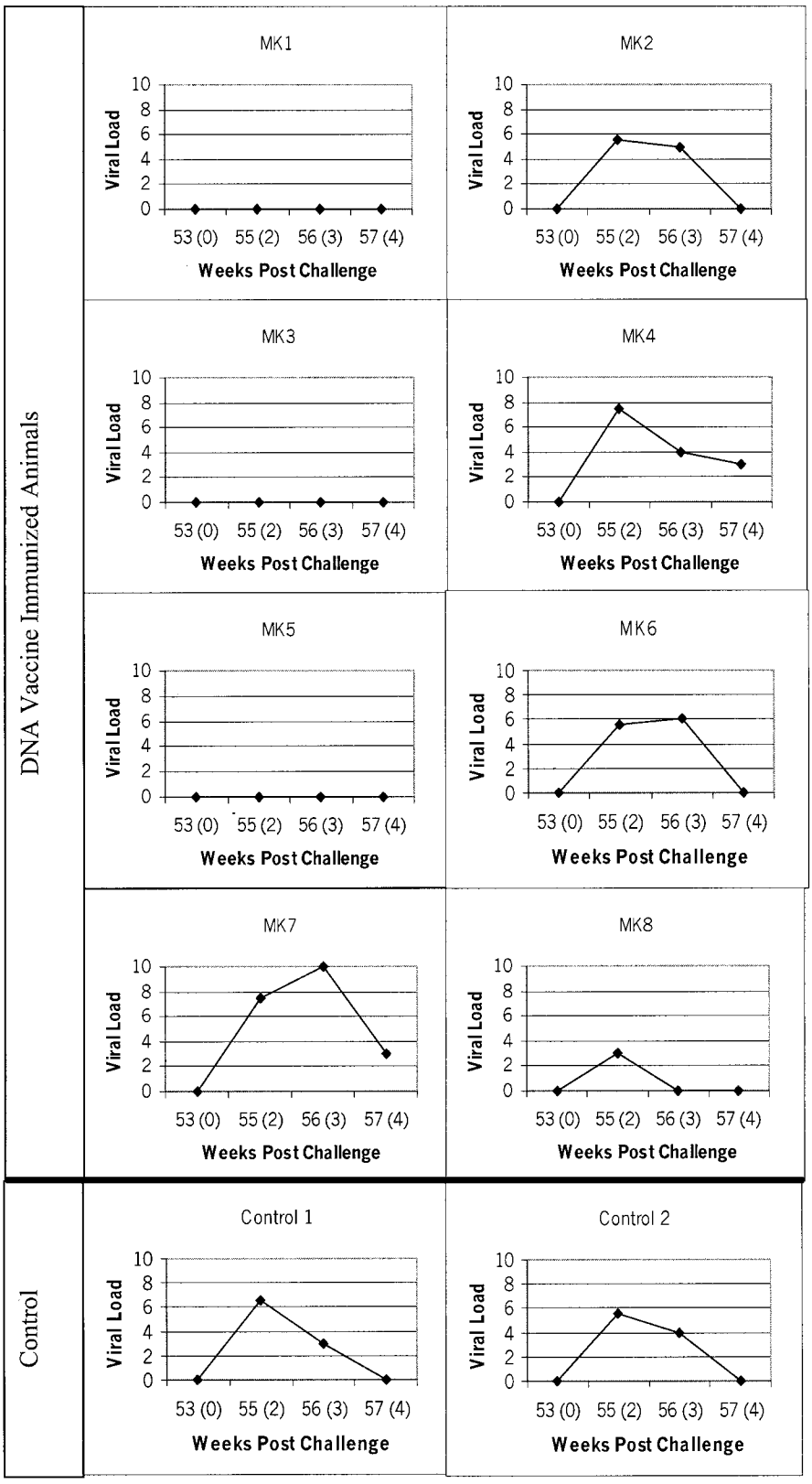


FIG. 1. Viral load following challenge with cell-free SHIV IIIB at week 53. The macaques were challenged by iv route with 10 animal infective doses (AID_{50}) of cell-free SHIV IIIB at week 53. Animals were then bled at 2, 3, and 4 weeks after the challenge and analyzed for virus detection using a sensitive limiting dilution coculture analysis.

TABLE 1

Cellular Responses in Protected vs Infected Rhesus Macaques

	Antigen-specific LPA responses (SI > 4)		
Protected	aEnv protein	aGag protein	Total
MK1	2/4	4/7	6/11
MK3	2/4	4/7	6/11
MK5	1/4	2/7	3/11
	5/12	10/21	15/33 (45%)
	Antigen-specific LPA responses (SI > 4)		
Infected	aEnv protein	aGag protein	Total
MK2	2/4	1/7	3/11
MK4	1/4	1/7	2/11
MK6	3/4	1/7	4/11
MK7	0/4	2/7	2/11
MK8	1/4	3/7	4/11
	7/20	8/35	15/55 (27%)

	No. of macaques with + CTL (>10%) at time of challenge (at week 53)
Protected	2/3 (67%)
Infected	2/6 (33%)

gag and gp130 env proteins. As shown in Fig. 2, after two booster immunizations (at weeks 81 and 85), all three immunized animals had positive LPA responses.

At week 89, these animals as well as a control macaque were challenged by iv route with 10 AID_{50} of pathogenic SIV_{mac239}. The animals were bled 2 and 4 weeks prior to challenge and at several time points following challenge. The infection with SIV_{mac239} was assessed for plasma antigenemia and by PCR analysis. Using a plasma antigenemia assay, we observed a high limiting dilution coculture level of p27 antigen in the plasma of the control animal at 2 weeks postchallenge, while we did not detect any p27 antigen in the three vaccine recipients (data not shown). These results were substantiated by the limiting dilution coculture and PCR analyses. The control animal was infected, as virus was readily detected within 1 week of challenge. The viral load remained high throughout the analysis period (greater than 10 logs of virus/10^6 cells). In contrast, 100% of the immunized macaques showed a complete absence of viral load when assayed by the limiting coculture method through 20 weeks postchallenge (Fig. 3A). The three animals were also virus-negative by PCR analysis.

Moreover, as early as 14 weeks post-SIV challenge, the $CD4^+$ and $CDw29^+$ T cell subsets in the control animal began to decline while the uninfected animals maintained normal levels of CD4 and CDw29 cell percentages (Fig. 3B). By 18 weeks, the infected control animal exhibited several adverse clinical symptoms such as weight loss, lethargy, ruffled fur, and diarrhea, consistent with SIV-induced disease. In fact, the health of the control animal continually deteriorated and the animal was euthanized by week 30 postchallenge. All vaccinated animals have remained healthy.

Since the design of the rechallenge study did not clearly demonstrate that the protection from SIV_{mac239} challenge is entirely due to DNA vaccines tested or due to DNA as prime and SHIVIIIB challenge as boost, we directly examined whether prior SHIVIIIB infection could provide protection from SIV_{mac239} challenge. A new set of eight rhesus macaques previously infected with SHIVIIIB virus was rested for 40 weeks after infection. We then rechallenged these animals as well as two naive control macaques with 10 AID_{50} of pathogenic SIV_{mac239}. As shown in Fig. 4, two naive control macaques were readily infected with SIV_{mac239} virus. We also observed that prior infection with SHIVIIIB virus did provide partial modulation of protection, as the SHIVIIIB infection lessened the viral load levels. On the other hand, prior infection with SHIVIIIB did not provide similar levels of protective immunity observed in vaccine-immunized rhesus macaques in Fig. 3, as only one of eight SHIVIIIB-infected macaques (12.5%) was protected from infection with SIV_{mac239} virus.

Protection from a pathogenic SHIV89.6P challenge

In order to further test this vaccination strategy against another pathogenic viral challenge, we sought to evaluate the effects of DNA vaccines against the severely pathogenic SHIV89.6P challenge model. A chimeric SHIV89.6P virus is a highly pathogenic and virulent virus generated by *in vivo* passages of SHIV89.6 virus in rhesus macaques (Karlsson *et al.,* 1997; Reimann *et al.,* 1996). SHIV89.6 virus is composed of SIVmac239 expressing HIV-1 tat, rev, vpu, and env of a cytopathic,

TABLE 2

Variant Levels of Cytokine and Chemokine Production by Protected and Infected Animals

	IFN-γ	IL-10	MIP-1α	MIP-1β	RANTES	MCP-1
Protected	662 (±20)	300 (±4)	4706 (±752)	12,642 (±3,512)	16,222 (±2,177)	6825 (±2,466)
Not protected	384 (±147)	286 (±35)	5905 (±1,331)	4988 (±2,091)	5851 (±2,678)	3633 (±1,392)
Student's *t* test (*P* value)	0.01	0.53	0.25	0.05	0.02	0.14

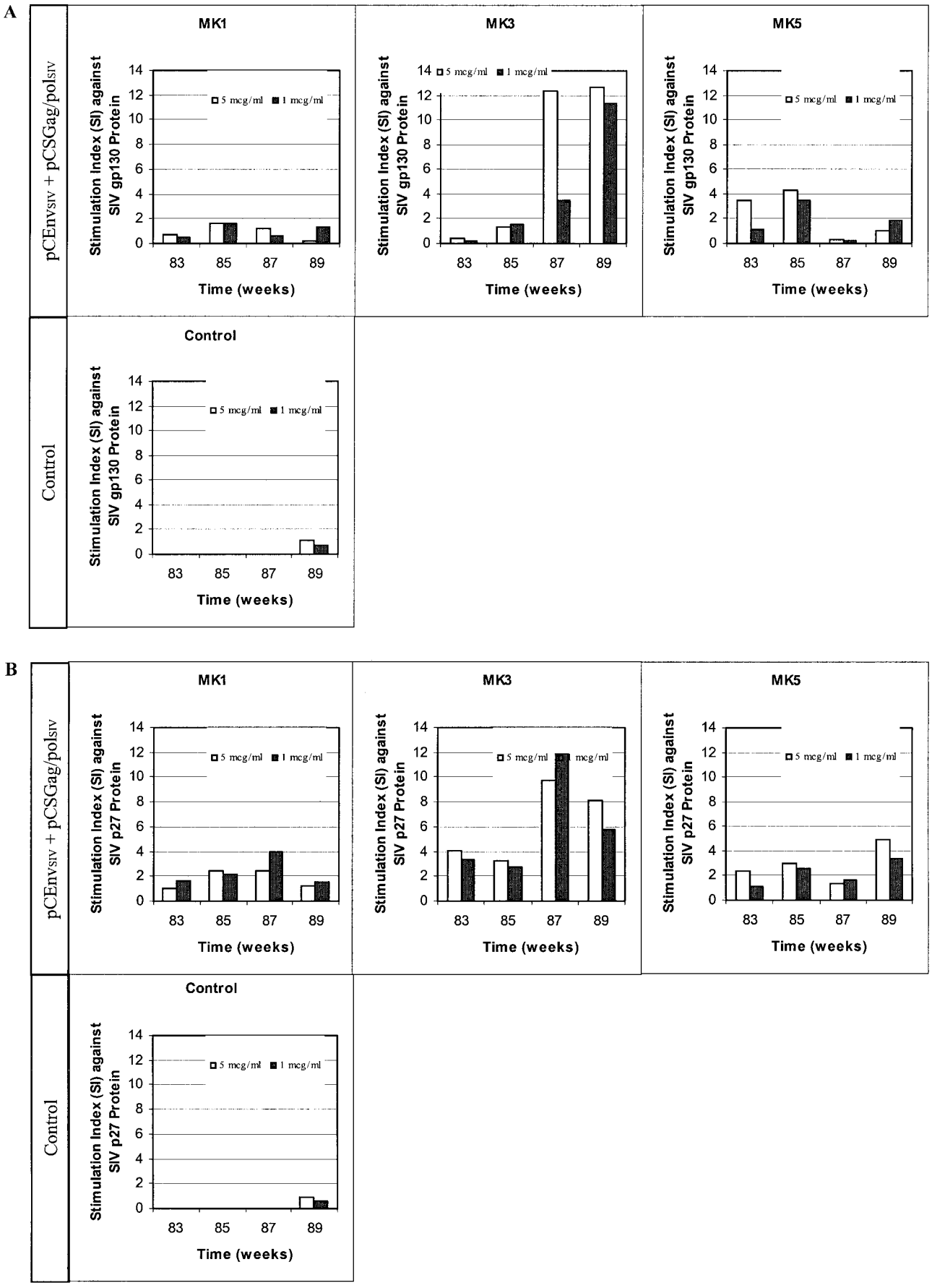
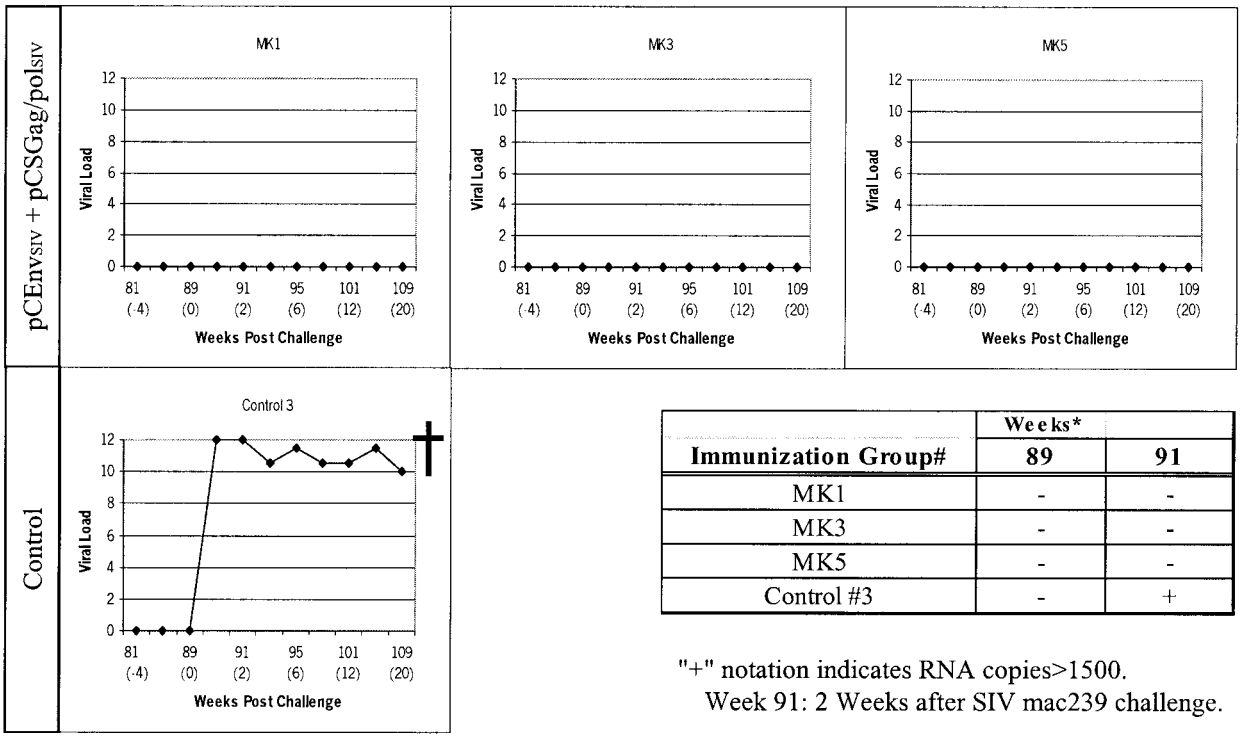


FIG. 2. Envelope-specific (A) and gag-specific (B) LPA responses following immunization and prior to pathogenic SIV_{mac239} challenge.

A



Immunization Group#	Weeks* 89	91
MK1	-	-
MK3	-	-
MK5	-	-
Control #3	-	+

"+" notation indicates RNA copies>1500.
Week 91: 2 Weeks after SIV mac239 challenge.

B

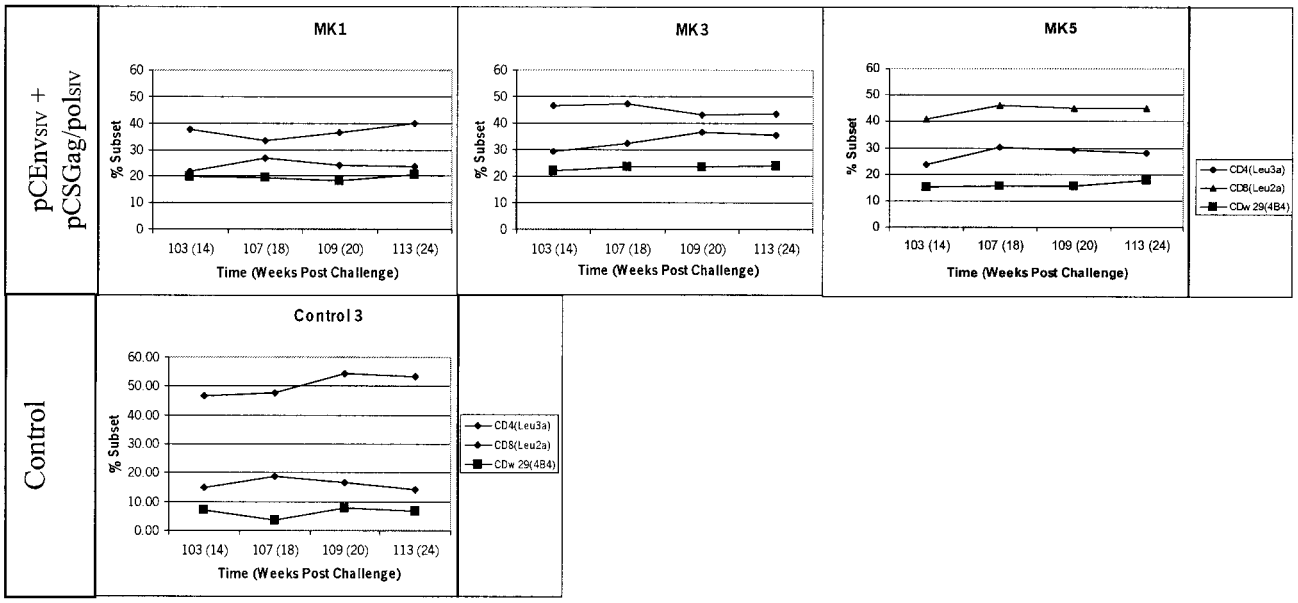


FIG. 3. (A) Viral load following challenge with pathogenic SIV_{mac239}. Three vaccinated animals as well as one control macaque were challenged by iv route with 10 AID_{50} of pathogenic SIV_{mac239} at week 89. Following the challenge with SIV_{mac239}, the animals were bled at several time points before and after the challenge and were analyzed for virus detection using a limiting dilution coculture analysis. The figure also shows the levels of SIV RNA in the plasma using the branched DNA assay before and after virus challenge. (B) Maintenance of $CD4^+$, $CD8^+$, and $CDw29^+$ cells following SIV_{mac239} challenge. Fourteen weeks following SIV challenge (week 103), whole blood samples were collected from the macaques and analyzed for T-lymphocyte subsets.

macrophage-tropic clone of a patient isolate of HIV-1 (89.6) (Reimann *et al.,* 1996). SHIV89.6P virus has been shown to induce rapid $CD4^+$ lymphocyte depletion, AIDS-like illness, and rapid disease in rhesus macaques (Reimann *et al.,* 1996, 1999).

The three protected monkeys from the SHIV IIIB and SIV_{mac239} challenges were rested for one additional year following the SIV_{mac239} challenge. These animals were boosted with 1 mg of HIV env_{MN}, a heterogeneous envelope, and SIV gag/pol DNA constructs at study weeks

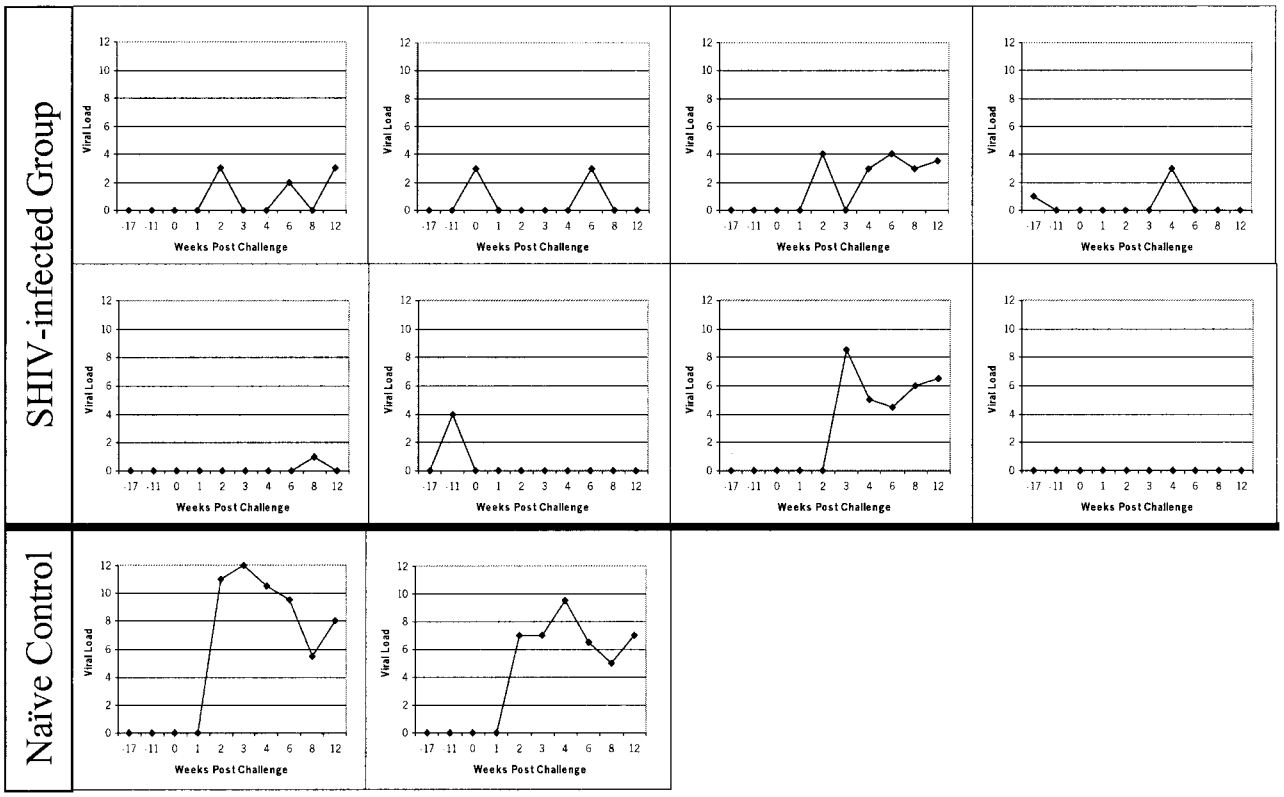


FIG. 4. Protective effects of SHIVIIIB infection on viral challenge with pathogenic SIV_{mac239}. Eight rhesus macaques infected with SHIVIIIB virus were rested for 40 weeks. These macaques as well as two naive control macaques were challenged by iv route with 10 AID_{50} of pathogenic SIV_{mac239}. Following the challenge with SIV_{mac239}, the animals were bled at several time points before and after the challenge and analyzed for virus detection using a limiting-dilution coculture analysis.

145 and 149. Subsequently, we analyzed the level of antigen-specific LPA responses against SIV p27 gag and HIV gp120 env_{MN} proteins. Throughout the analysis period, all three immunized animals showed potent antigen-specific LPA responses to SIV p27 gag and HIV gp120 env_{MN} proteins, while the control animal did not show any reactivity against the gag and env antigens (Fig. 5). It is also important to note that the animals were immunized with DNA construct expressing HIV envelope protein from the MN strain and not from the 89.6P strain. We also analyzed the IFN-γ expression profiles from the animals immediately prior to challenge at week 153. As shown in Fig. 6C, we observed higher levels of IFN-γ from the immunized rhesus macaques versus the control animal. These results are consistent with the results shown in Table 2, where the stimulated T cells from the rhesus macaques protected from SHIVIIIB produced higher levels of IFN-γ than the control or immunized but unprotected animals.

At week 153, these animals as well as a control naive macaque were challenged by the iv route with 10 AID_{50} of pathogenic SHIV89.6P. Blood was drawn from the animals several weeks prior to challenge and at time points following challenge. Infection by SHIV89.6P was assessed by limiting dilution coculture and PCR analysis. We observed a high level of viral infection in the control animal within 1 week of challenge, and the viral load remained high throughout the analysis period. In contrast, 100% of the immunized macaques showed absence of viral load when assayed by the limiting coculture method through 6 weeks postchallenge (Fig. 6A). The immunized animals were also virus-negative by PCR analysis.

T cell subsets were also studied. Compared to the control animal from the SIV_{mac239} challenge, the control animal from the SHIV89.6P challenge had its $CD4^+$ and $CDw29^+$ T cell subsets decline much more rapidly (Fig. 6B). As early as 2 weeks post-SHIV89.6P challenge, the $CD4^+$ and $CDw29^+$ T cell subsets on the control animal began to collapse, while the immunized and uninfected animals maintained normal levels. The infected control animal exhibited severe adverse clinical symptoms such that the animal had to be euthanized by week 24 postchallenge. All vaccinated animals have remained healthy and viral negative and were protected from T cell aspects of viral pathogenesis during the duration of observation (>1 year postchallenge) (data not shown).

DISCUSSION

One of the hallmarks of HIV-1 disease progression is the loss of cellular immune function, and the presence of strong cellular responses in some instances can correlate with control of viral replication (Kim and Weiner,

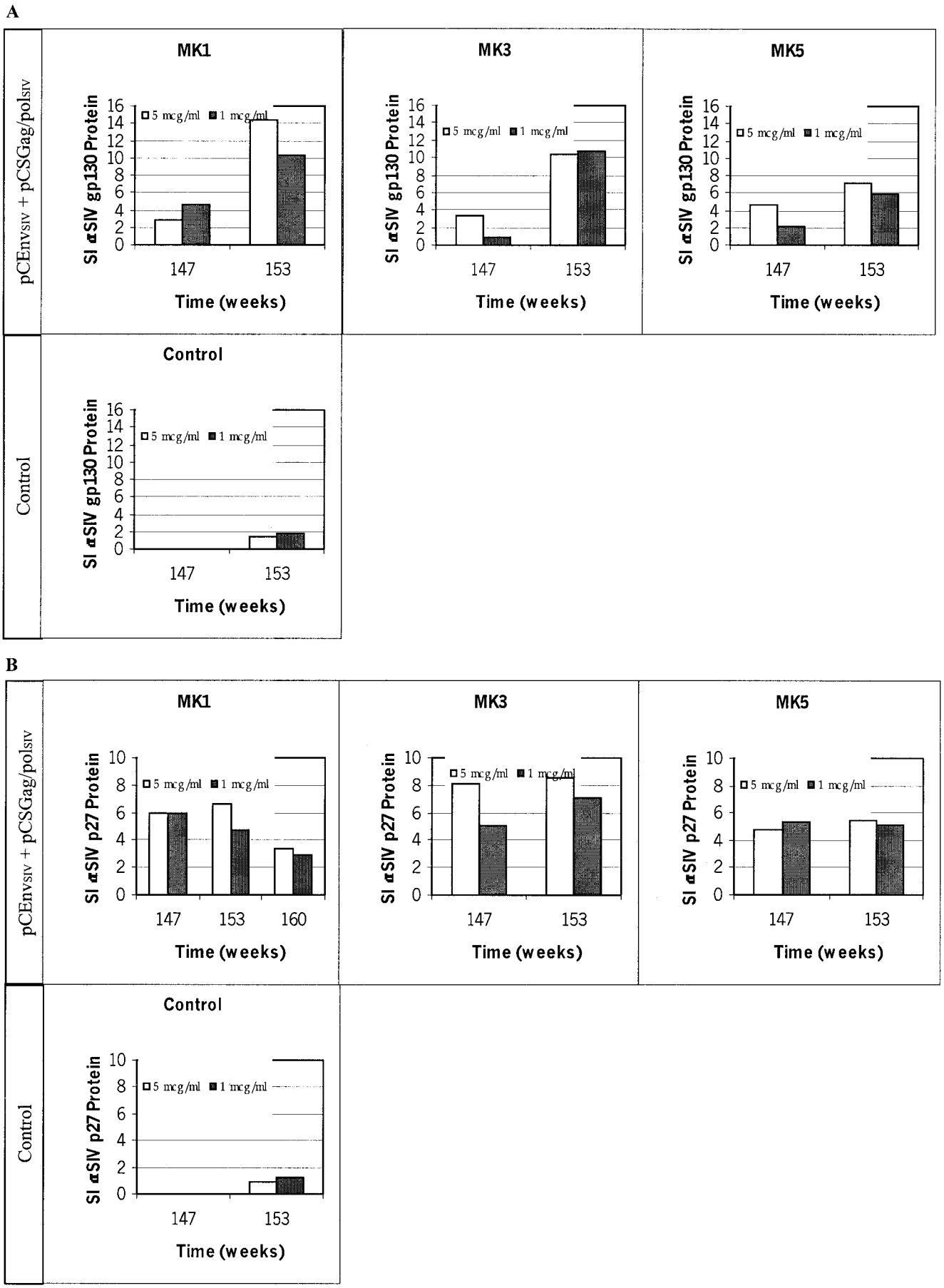
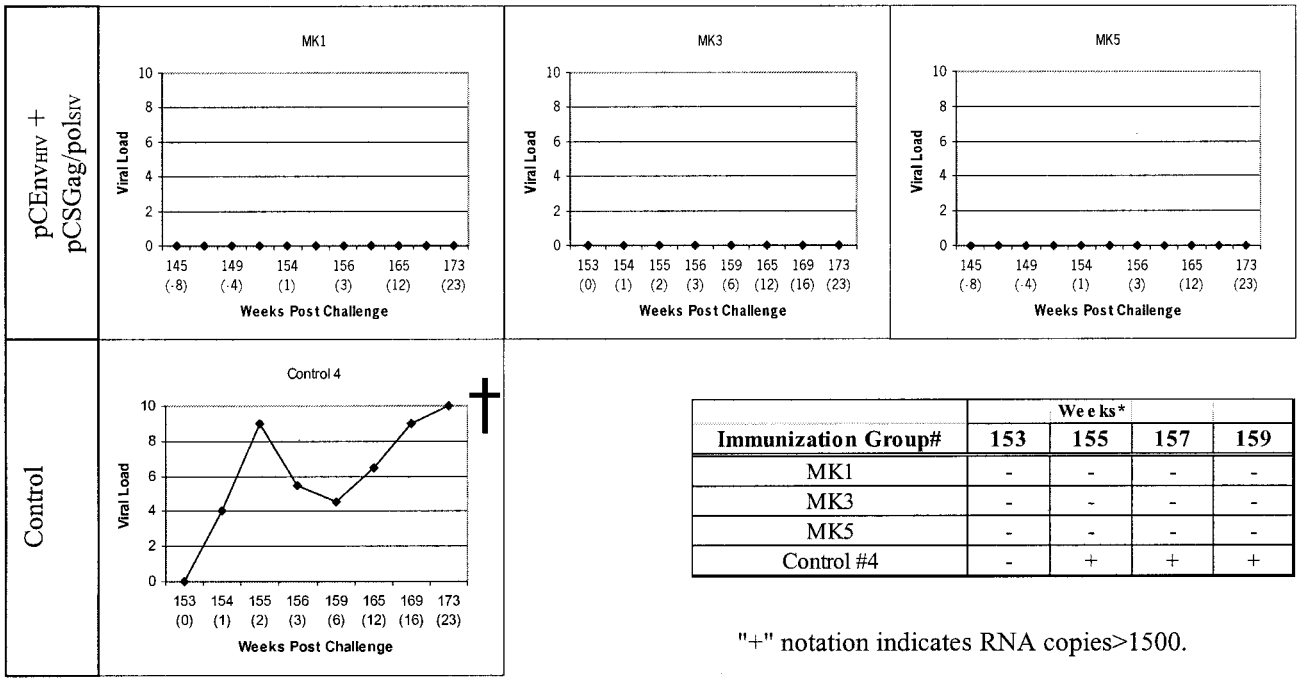


FIG. 5. Envelope-specific (A) and gag-specific (B) LPA responses following immunization and prior to pathogenic SHIV89.6P challenge.

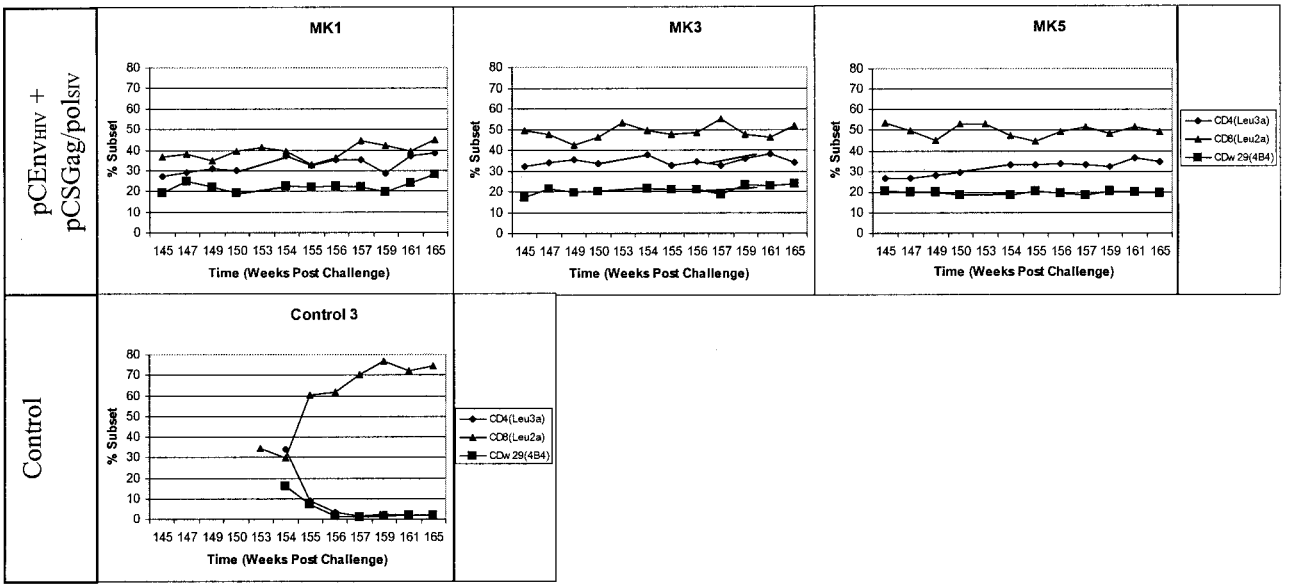
A



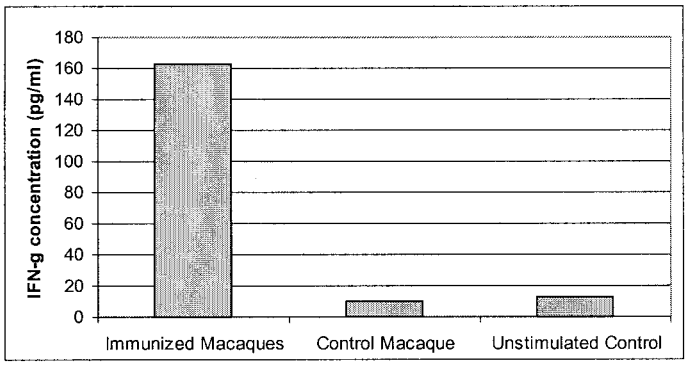
Immunization Group#	Weeks* 153	155	157	159
MK1	-	-	-	-
MK3	-	-	-	-
MK5	-	-	-	-
Control #4	-	+	+	+

"+" notation indicates RNA copies>1500.

B



C



1997; Letvin, 1998). In cases of acute HIV-1 infection studied by several investigators, viral clearance was associated with specific CTL activity in each case (Borrow *et al.,* 1994; Koup *et al.,* 1994). In addition, a subset (7 of 20) of occupationally exposed health-care workers who were not infected possessed transient HIV-1 specific CTL response (Pinto *et al.,* 1995). HIV-1-specific CTLs were also found in a number of chronically exposed sex workers in Gambia who continue to resist infection with HIV-1 (Rowland-Jones *et al.,* 1995). In spite of these studies supporting the role of neutralizing antibodies and CTLs in conferring immunity to infection, some vaccinated primates exhibiting both neutralizing antibody and CTL responses were not protected from subsequent viral challenge in the pathogenic SIV model (Hulskotte *et al.,* 1995). Recently, the important role of CD4 helper responses in the anti-HIV immune response has been highlighted (Rosenberg *et al.,* 1997). Such responses likely have importance for both humoral and $CD8^+$ effector responses.

Overall, the present studies provide some insights into the importance of T-cell-mediated immune responses. Neutralizing antibodies were not detected at the time of SHIVIIIB challenge (Kim *et al.,* 2000) and the envelope constructs used in the first and third challenges were not matched. Here, the results from the three SHIV and SIV challenge model studies support the finding that CMI responses, especially the Th1-type T cell responses, might be important for protection against infectious and pathogenic challenges. In these studies, the protective effects seemed to suggest the presence and the strength of antigen-specific LPA responses. Furthermore, these LPA responses had Th1 phenotype, with potent induction of IFN-γ. For instance, the level of IFN-γ detected in lymphocytes from the macaques protected from the SHIVIIIB challenge was greater than those of the unprotected control animals or the unprotected vaccinated animals (Table 2). Similarly, the macaques protected from the SHIV89.6P challenge had high levels of T-cell-produced IFN-γ (Fig. 6C). The data also support further investigation of the importance of T-cell-produced chemokines. The protected rhesus macaques also produced significantly higher levels of MIP-1β (P value = 0.05) and RANTES (P value = 0.02) than the unprotected animals (Table 2). These results support the finding that induction of β-chemokines through an immunization strategy should be further studied in relation to protection (Heeny *et al.,* 1997; Lehner *et al.,* 1996).

Accordingly, one possible correlate appears to be driving responses toward a Th1 phenotype, a theory that had been under investigation previously (Clerici *et al.,* 1993). This current study tested a low-dose immunization protocol compared to doses in other studies (Letvin *et al.,* 1997; Lu *et al.,* 1996; Putkonen *et al.,* 1998). It will be important to evaluate more aggressive doses and reexamine protection in a larger number of animals. The protective results against both SHIV and SIV challenges strongly suggest an important role for gag/pol immunogens in generating protective responses in these models and the development of vaccines in humans.

As it has already been clearly established that env protein vaccines can protect in SHIVIIIB model in a type-specific fashion, these results support the finding that multiple immune mechanisms can be important and together may broaden protection (Letvin *et al.,* 1997). In the present trials this appears not to be the case as no clear induction of type-specific neutralizing antibodies were observed in protected animals in any of the studies. Furthermore, in the latter challenge, MN env-immunized animals were protected from iv challenge with SHIV 89.6P virus, further distinguishing the protection observed here from a type-specific antibody response. In this pilot study, we observed that the animals protected in the first study were protected in subsequent challenges with SIV_{mac239} and SHIV89.6P. Animals were protected based on a lack of antigenemia, viral detection, and most importantly, disease and death in all immunized macaques, strongly supporting additional studies in this area. The results are important as they support the finding that cellular immune responses can play a role in this important outcome. However, these studies only support and do not clearly establish which cellular arms are responsible for viral control in this model.

It was not clear whether the protection from SIV_{mac239} challenge is entirely due to DNA vaccines tested or due to DNA as prime and SHIVIIIB challenge as boost. More specifically, it is important to consider the role of SHIVIIIB challenge as a boosting agent for gag-specific cellular responses in this study. It has been previously reported that prior infection with a SHIV HXBc2 virus does not provide protective immunity against a pathogenic SIV challenge (Letvin *et al.,* 1995). In this regard, we directly examined whether prior SHIVIIIB infection could provide protection from SIV_{mac239} challenge. Eight rhesus macaques infected with SHIVIIIB virus were rested for 40 weeks. We then rechallenged these animals as well as two naive control macaques with 10 AID_{50} of pathogenic SIV_{mac239}. As shown in Fig. 4, two naive control macaques

FIG. 6. (A) Viral load following challenge with pathogenic SHIV89.6P. Three vaccinated animals as well as one control macaque were challenged by iv route with 10 AID_{50} of pathogenic SHIV89.6P at week 153. Following the challenge with SHIV89.6P, the animals were bled at several time points before and after the challenge and were analyzed for virus detection using a dilution coculture analysis. The figure also shows the levels of SIV RNA in the plasma using the branched DNA assay before and after virus challenge. (B) Maintenance of $CD4^+$, $CD8^+$, and $CDw29^+$ cells following SHIV89.6P challenge. The animals were bled at several time points before and after the challenge and their whole blood samples were analyzed for T-lymphocyte subsets. (C) Blood samples were drawn from animals immediately prior to challenge at week 153. Purified PBMC were stimulated *in vitro* for 72 h with SIV gag p27 protein (at 5 μg/ml). Expression of IFN-γ from stimulated T cells were analyzed by ELISA.

were readily infected with SIV_{mac239} virus. We observed that prior infection with SHIVIIIB virus did not provide similar levels of protective immunity observed in Figs. 3 and 6, as only one of eight SHIVIIIB-infected macaques (12.5%) was protected from infection with SIV_{mac239} virus. Previous infection did seem to provide partial modulation of protection as the SHIVIIIB infection lessened the viral load levels (Fig. 4).

It is also interesting that studies from Rupprecht *et al.* support the finding that a threshold of replication is required to engender subsequent protection (Ruprecht *et al.,* 1996). This was not the case in our studies, which resulted in protection from SIV_{mac239} and SHIV89.6P challenges, as no clear levels of replication were observed.

A study by Robinson *et al.* is important in this regard using a prime (ID DNA) boost (fowlpox) approach followed up by nonpathogenic SHIV and then subsequent pathogenic SHIV challenge (Robinson *et al.,* 1999). These authors also found little evidence of neutralizing antibody response-mediated protection. The results of our study are highly consistent with these concepts. In addition, it was observed that cellular responses can have a direct protective role against pathogenic and nonpathogenic challenges. Importantly, new insight provided by these challenges extends these properties, perhaps under certain conditions, to im DNA vaccination in the absence of live vector boosting. These studies are also supported by the recent work of Barouch *et al.* (2000). In this study, plasmid vaccines encoding env and gag from 89.6P virus and IL-2 Ig either as protein or as plasmid did not prevent infection but prevented significant loss of CD4 T cells and resulted in animals that could control viral load. These studies, however, used unmatched envs for the challenge, so the questions of humoral responses in protection were unresolved. The observation in two separate studies that protection in nonpathogenic SHIV model systems results in successes with high frequency in pathogenic challenges should not go unappreciated.

Additional studies are warranted to resolve these issues. However, these results demonstrate that protection from pathogenic challenge can be achieved in the absence of high viral replication that reaches a threshold level of replication for effective vaccination (Figs. 3A and 6B), a concern for vaccine safety (Ruprecht *et al.,* 1996). Furthermore, significant control over viral set point and prevention of CD4 loss, disease, and death (Figs. 3B and 6B) can be achieved in multiple nonhuman primate challenge models of HIV through such an immunization approach. This is encouraging for further development of a prophylactic vaccine for HIV-1, as it implies that viral set point in this model can be controlled with a combination of vaccination techniques which are conceptually simple to design and likely to be safe to administer. However, ultimately, the use of primate models to predict effectiveness in human population is of considerable debate.

MATERIALS AND METHODS

DNA plasmids

DNA vaccine constructs expressing HIV-1 envelope protein (pCEnv) and those expressing SIV gag/pol protein (pCSGag/Pol) were prepared as previously described (Wang *et al.,* 1995). The cytokine genes were cloned into the pCDNA3 expression vector (Invitrogen, Inc., San Diego, CA) as previously described (Kim *et al.,* 1998b).

Reagents

Recombinant HIV-1 gp120 and SIV p27 proteins were obtained from ImmunoDiagnostics, Inc. (Bedford, MA).

Animals

Rhesus macaques (*Macaca mulatta*) were individually housed at the Primedica Mason Labs (Worcester, MA). All animal care and use procedures conformed to the revised Public Health Service Policy on Humane Care and Use of Laboratory Animals. Animals were anesthetized with ketamine HCl for all technical procedures.

Macaque immunization and challenge virus inoculation

Macaques were immunized intramuscularly (im) in the quadriceps with DNA preparations formulated in phosphate-buffered saline (PBS) and 0.25% bupivacaine–HCl (Sigma, St. Louis, MO) (Kim *et al.,* 1997, 1998b) on multiple occasions. Initially, five groups of two rhesus macaques each were immunized with various DNA vaccine constructs. The first group (MK1 and MK2) was immunized with HIV env_{MN}/rev (pCEnv) and SIV gag/pol (pCSGag/pol) constructs along with a control vector pCDNA3. The second group (MK3 and MK4) was immunized with pCEnv + pCSGag/pol + IL-2 constructs. The third (MK5 and MK6) and fourth groups (MK7 and MK8) were immunized with pCEnv + pCSGag/pol + IL-4 and pCEnv + pCSGag/pol + IFN-γ, respectively. These macaques were immunized with 200 μg of each DNA at weeks 0, 6, and 12 and boosted with 500 μg of each DNA at weeks 28 and 49. These constructs were mixed prior to injection into the quadriceps muscle. The control group was immunized with 500 μg of pCDNA3 at weeks 28 and 49. At week 53 of the study, all macaques were challenged intravenously (iv) with 10 AID_{50} of SHIV IIIB (provided by Yichen Lu, Virus Research Institute). At week 89, a subset of animals that were negative for virus recovery following SHIV challenge were challenged iv with 10 AID_{50} of SIV_{mac239} (provided by Ronald C. Desrosiers, New England Regional Primate Research Center). Naive control animals were included at each challenge time point. At week 153, three immunized macaques and one control macaque were challenged iv with 10 AID_{50} of pathogenic SHIV89.6P (provided by Norman Letvin, Harvard Medical School).

Lymphoproliferative assay

Peripheral blood lymphocytes were prepared as previously described (Boyer *et al.,* 1997). The isolated cell suspensions were resuspended to a concentration of 5×10^5 cells/ml in a medium consisting of RPMI 1640 (Gibco-BRL, Grand Island, NY) with 10% fetal calf serum (Gibco-BRL). A 100-μl aliquot containing 5×10^5 cells was immediately added to each well of a 96-well microtiter round-bottom plate. Recombinant p27 or gp120 protein at final concentrations of 5 and 1 μg/ml were added to wells in triplicate. The cells were incubated at 37°C in 5% CO_2 for 3 days. One microcurie of tritiated thymidine was added to each well and the cells were incubated for 12–18 h at 37°C. The plates were harvested and the amount of incorporated tritiated thymidine was measured in a Beta Plate reader (Wallac, Turku, Finland). Stimulation Index was determined from the formula

Stimulation Index (SI)

= (experimental count/spontaneous count).

Spontaneous count wells (media only) include 10% fetal calf serum. To ensure that cells were healthy, concanavalin A (Sigma) was used as a polyclonal stimulator positive control.

Cytotoxic T-lymphocyte assay

A standard 5-h ^{51}Cr-release CTL assay was performed on PBMCs from the inoculated and control macaques as previously described (Boyer *et al.,* 1997). Cells for *in vitro* stimulation of T cells were prepared by infecting autologously transformed B-lymphoblastoid cell lines (LCLs) with a recombinant vaccinia virus which expressed SIV_{mac239} env/gag/pol proteins (Therion, Boston, MA). Prior to use the infected cells were fixed with 0.1% glutaraldehyde and blocked with a 0.1 mM glutamine solution. The fixed cells were incubated with the isolated PBMCs (effectors) for stimulation in CTL stimulator medium (RPMI 1640 (Gibco-BRL), 10% fetal calf serum (Gibco-BRL), and recombinant IL-2 (40 U/ml) (Intergen, Purchase, NY)) for 21 days. The LCLs infected with specific recombinant vaccinia virus or control recombinant vaccinia virus expressing β-galactosidase (vSC8) were also used as target cells. Cells incubated with the control vaccinia were used as targets to provide background levels of lysis.

Cytokine and chemokine expression analysis

Supernatants from effectors stimulated for CTL assay (see above) were collected after specific stimulation and were tested for cytokine profile using ELISA kits for IFN-γ and IL-10 (Biosource International, Inc., Camarillo, CA). Similarly, expression of MIP-1α, MIP-1β, RANTES, and MCP-1 was analyzed using ELISA kits (Intergen). For each sample at each time point, 100 μl of supernatant was tested in triplicate wells. Each well value was used to derive the average and the standard deviation values. The *P* values were calculated using Student's *t* test.

Cell-associated virus load by limiting dilution coculture

Viral load was determined by limiting dilution coculture of isolated PBMC with CEMx174 target cells using a method previously described (Wyand *et al.,* 1996). Twelve serial 1:3 dilutions of PBMC, beginning with 10^6 cells, were cocultured in duplicate with 10^5 CEMx174 cells per well in 24-well plates. Supernatant samples were collected after 21 days of culture and stored frozen at −70°C until analysis for p27 antigen with the Coulter p27 antigen assay kit.

Plasma RNA

Plasma samples from whole blood collected in sodium citrate were analyzed for SIV RNA copies per milliliter using the branched DNA assay (bDNA) developed by Chiron Corp., Emeryville, CA.

Flow cytometry

Whole blood collected in EDTA was analyzed for lymphocyte subsets CD4 (Anti-Leu 3a (Becton–Dickinson)), CD8 (Anti-Leu 2a (Becton–Dickinson)), and CDw29 (4B4) (Coulter Immunology) after red blood cell lysis using methods previously described (Wyand *et al.,* 1996). Briefly, antibody (volume dependent upon antibody) was added to 100 μL of whole blood and incubated for 10 min in the dark. Lysing solution (Becton–Dickinson) was added and the samples were incubated for 10 min at room temperature. Stained cells were fixed with 0.5% paraformaldehyde. Samples were analyzed on a Becton–Dickinson FACScan cytometer.

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