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Improved Vaccine Protection from Simian AIDS by the Addition of Nonstructural Simian Immunodeficiency Virus Genes

Zdeněk Hel,*+ Wen-Po Tsai,* Elżbieta Tryniszewska,** Janos Nacsa,† Phillip D. Markham,§ Mark G. Lewis,‖ George N. Pavlakis,‖ Barbara K. Felber,‡ Jim Tartaglia,** and Genoveffa Franchini*‡

An HIV-1 vaccine able to induce broad CD4+ and CD8+ T cell responses may provide long-term control of viral replication. In this study we directly assess the relative benefit of immunization with vaccines expressing three structural Ags (Gag, Pol, and Env), three early regulatory proteins (Rev, Tat, and Nef), or a complex vaccine expressing all six Ags. The simultaneous administration of all six Ags during vaccination resulted in Ag competition manifested by a relative reduction of CD8+ T cell and lymphoproliferative responses to individual Ags. Despite the Ag competition, vaccination with all six Ags resulted in a delay in the onset and a decrease in the extent of acute viremia after mucosal challenge exposure to highly pathogenic SIVmac251. Reduced levels of acute viremia correlated with lower post-set point viremia and long-term control of infection. In immunized animals, virus-specific CD4+ T cell and lymphoproliferative responses were preserved during acute viremia, and the maintenance of these responses predicted the long-term virological outcome. Taken together, these results suggest that the breadth of the immune response is probably more important than high frequency responses to a limited number of epitopes. These data provide the first clear evidence of the importance of nonstructural HIV Ags as components of an HIV-1 vaccine. The Journal of Immunology, 2006, 176: 85–96.

Humun immunodeficiency virus type 1/SIV infection is associated with acute loss of CD4+ T cells (1–3), a progressive demise of the immune system, and AIDS (4, 5). Although the host mounts strong cellular and humoral immune responses to HIV/SIV, the impairment of CD4+ T cells help, the functional exhaustion of CD8+ T cells, and the plasticity of the viral genome contribute to the inefficient control of viral replication and consequent disease progression. Thus, effective T cell vaccines for HIV/SIV should elicit CD4+ and CD8+ T cell responses of sufficient breadth and size to minimize viral replication and immune escape. Promising approaches to vaccine design have included combinations of heterologous vaccine modalities, because they appear to provide superior protection against HIV/SIV infection. Among these, the DNA/poxvirus combination has been demonstrated to be immunogenic in macaques and confer a degree of protection that depends on the dose and type of DNA, the level of expression of HIV/SIV Ags by the poxvirus vector, and the challenge virus used (6–11). In most of those studies, structural HIV/SIV Ags have been used, and the contribution of nonstructural Ags to protection remained unclear because head-to-head comparisons in a relevant animal model have not been performed.

The HIV/SIV genome encodes the structural/enzymatic proteins Gag, Pol (reverse transcriptase, protease, and integrase), and Env and the regulatory proteins Rev, Tat, Nef, Vif, Vpx, and Vpr. HIV-1 encodes an additional protein, Vpu, but lacks the open reading frame for Vpx. After viral entry, reverse transcription, and integration of the viral genome, the first genes expressed by HIV/ SIV are completely spliced mRNAs that encode for the nonstructural Tat, Rev, and Nef proteins (12–14). Only when Rev reaches a sufficient level are the incompletely splicedEnv mRNA and the genomic RNA encoding for Env and Gag-Pol, respectively, transported to the cytoplasm for translation. Being expressed early in the viral life cycle, Tat, Rev, and Nef proteins may be recognized by the immune system early in infection. Indeed, cytotoxic responses to epitopes encoded by these open reading frames are frequently detected in primary HIV infection (15, 16). Immune response to early regulatory genes may be particularly important to viral containment, because their recognition by cytotoxic CD8+ T cells may occur before Nef-mediated down-modulation of MHC class I molecules on the surface of infected cells, resulting in the elimination of infected cells before the release of virions (17–22). The ability of early regulatory protein-specific CTLs to exert immunological pressure has been inferred by the selection for viral immune escape variants in Tat (23) and Nef (24, 25) during primary SIV infection. In both macaques and humans, the presence of humoral and cellular immune responses against Tat (26–30) and of CTL responses against Rev (31, 32) and Nef (33) has been demonstrated to inversely correlate with disease progression.

The inclusion of regulatory proteins as part of vaccines against immunodeficiency viruses has been tested in both macaques and humans with variable results (34–43). Immunization with Tat alone or a Tat toxoid, for example, has conferred limited or no
protection depending on the animal model and challenge virus (34, 35, 41, 44).

The use of unaltered tat, rev, and nef gene products as components of a vaccine candidate has possible limitations due to the intrinsic ability of early regulatory proteins to interfere with host immune response mechanisms by various mechanisms, including down-modulation of MHC class I and II, CD4, and CD28 proteins; induction of T cell unresponsiveness and apoptosis; and up-regulation of Fas ligand (17, 45–52). Furthermore, the small size of these proteins represents a limitation, because fewer epitopes have a chance to be recognized by a given MHC class I. In addition, the expression of HIV and SIV genes in mammalian cells has proven difficult because of a highly distinct codon bias for adenine and thymidine at the third codon position (53, 54), limiting their translational efficiency. To overcome the potential shortcomings of early gene-based vaccines, we designed a novel chimeric gene comprising genetically modified, attenuated, and reassorted rev, tat, and nef genes of SIV (retanef) that has been optimized for expression in mammalian cells (55).

To assess directly the contribution of nonstructural proteins to vaccine protection, we have performed a head-to-head comparison of the protective effect of DNA- and NYVAC-based vaccines expressing the chimeric Retanef protein alone (55), a combination of Gag, Pol, and Env proteins (56), or a combination of all six Ags together in the SIVmac251 macaque model. We used a DNA prime/poxvirus boost regimen with highly attenuated NYVAC-based recombinant SIV vaccines, because we have previously demonstrated that the regimen confers higher frequency of virus-specific CD4+ and CD8+ memory T cell responses than recombinant NYVAC-SIV alone (8, 9). In this study we demonstrate for the first time that the addition of modified early SIV regulatory Ags to structural SIV Ags results in a delay of primary viremia, reduces its extent, preserves virus-specific CD4+ T cells, and increases survival of the immunized animals. In addition, the data presented in this study suggest that the simultaneous administration of multiple Ags in primates may result in Ag competition and decreased immune responses to individual Ags.

Materials and Methods

Animals, immunizations, and challenge

All animals were colony-bred rhesus macaques (Macaca mulatta) obtained from Covance Research Products. The animals were housed and handled in accordance with the standards of the Association for the Assessment and Accreditation of Laboratory Animal Care International, and the study was reviewed and approved by the animal care and use committees at Advanced BioScience Laboratories and Bioqual. All rhesus macaques were seronegative for SIV-1, simian T cell leukemia/lymphoma virus type 1, and hepatitis B virus before the study. Molecular typing of MHC class I alleles that bind SIV-derived peptides, namely, Mamu-A*01, Mamu-A*02, Mamu-A*08, Mamu-A*11, Mamu-B*01, Mamu-B*03, Mamu-B*04, Mamu-B*17, and Mamu-B*29, was performed using the technique of sequence-specific primer DNA amplification as previously described (57–59). The 3′-terminal region of sequence-specific primer DNA amplifications targeted nucleotide polymorphism unique to rhesus MHC class I alleles. Large scale, low endotoxin plasmid DNA was prepared by Qiagen under good manufacturing practices specifications. All NYVAC virus-derived preparations were provided by Sanofi-Pasteur.

Group 1A animals were not immunized. The animals in control group 1B were immunized with 108 PFU of mock NYVAC at weeks 0, 4, 24, and 52. At weeks 0, 4, and 12, animals in groups 2, 3, and 4 were immunized with plasmid DNA in PBS (1 mg/ml) administered by i.m. and intradermal routes. SIV-Retanef (55) and Rev-independent SIV Gag and SIV Env (8) expression vectors optimized for high level expression in primate cells were described previously. Group 2 received 1 mg of Retanef-encoding plasmid (five doses of 0.2 ml each) intradermally into five different sites in the abdominal area and 1 mg of Retanef (four doses of 0.25 ml each) i.m. into two sites on each leg. For the third immunization, the dose of Retanef administered i.m. was increased to 3 mg (four doses of 0.75 ml each). Group 4 received a mix of 1 mg of Env and 1 mg of Gag plasmids (10 doses of 0.2 ml each) intra-dermally into 10 different sites in the abdominal area and 3 mg of Env and 3 mg of Gag (four doses of 1.5 ml each) i.m. into two sites on each leg. Group 3 was immunized with both Retanef and Env/Gag plasmids, as described for groups 2 and 4. At weeks 24 and 52, groups 2, 3, and 4 were boosted with 106 PFU of the appropriate NYVAC recombinant vaccines given i.m.; group 4 received NYVAC-SIV-gag-pol-env (NYVAC-SIV-gpe), group 2 received NYVAC-SIV-retanef (NYVAC-SIV-rtn), and group 3 received both vaccines at the same dose (see Fig. 1).

The challenge virus stock was prepared from PHA-activated PBMCs obtained from Mamu-A*01-positive macaque 561L, previously inoculated vaginally with SIVmac251 (59). The monkeys were challenged by intrarectal exposure to 30 mucosal infectious doses of SIVmac251 challenge stock. SIVmac251 RNA in plasma was quantified by nucleic acid sequence-based amplification (60). Data describing the immunization and challenge of animals in groups 1B and 4 were published previously (8, 9). Because the same batches of DNA, recombinant NYVAC viruses, and challenge virus as well as identical immunological and virological assays were used in all studies, data from previous experiments were used for comparison purposes in the current study.

Lymphocyte proliferation assay

The Ficol1-purified PBMCs were resuspended in RPMI 1640 medium (In-vitrocel Life Technologies) containing 5% inactivated human A/B serum and antibiotics (Sigma-Aldrich), and cultured at 106 cells/well in triplicate for 3 days in the absence or the presence of native HPLC-purified SIVmac Gag p27 or Env gp120 proteins (Advanced BioScience Laboratories) or Con A as a positive control. The cells were then pulsed overnight with 1 µCi of [3H]thymidine before harvest. The relative rate of lymphoproliferation was calculated as the fold thymidine incorporation into cellular DNA over medium control (stimulation index).

Detection of epitope-specific CD4+CD8+ T lymphocytes by tetramer staining

Fresh PBMCs were stained with anti-human CD3 and CD8a Abs (BD Pharmingen) and Mamu-A*01 tetrameric complexes (provided by Dr. I. Altman, Emory University Vaccine Center at Yerkes, Atlanta, GA) re-folded in the presence of a specific peptide and conjugated to PE-labeled streptavidin (Molecular Probes). Gag181–189 CM9 (CTPYDINQM; Gag_CM9) and Tat28–35 SL8 (TTPESANL; Tat_SL8)-specific tetramers were used. Samples were analyzed on FACSCalibur (BD Biosciences), and the data are presented as the percentage of tetramer-positive cells of all CD3+CD8+ lymphocytes.

Intracellular cytokine (ICC) staining

SIV-specific CD4+ and CD8+ T cell responses were detected using pools of 15-meric peptides overlapping by 11 amino acids covering entire Gag, Rev, Tat, and Nef proteins of SIVmac251. Cells (1 × 106) in RPMI 1640 medium (containing 10% human serum and antibiotics) were incubated in the absence or the presence of a specific peptide pool at 2 µg/ml of each peptide for 1 h as previously described (61). Brefeldin A (Sigma-Aldrich) at a final concentration of 10 µg/ml was added, and the cells were incubated for an additional 5 h. The cells were washed, stained for the surface Ags CD3e and CD8a, permeabilized by incubation in FACSSperm solution, and stained with anti-TNF-α-FITC, anti-IFN-γ-FITC Abs (all reagents and Abs from BD Pharmingen). The results were calculated as the total number of cytokine-positive cells with background subtracted.

Statistical analysis

All reported p values are two-sided. All correlation coefficients were calculated using the Spearman rank-order correlation test with a 95% confidence interval. Viral loads were compared by the Mann-Whitney rank-sum test. The Number Cruncher Statistical System (NCSS) and SigmaStat (version 2.0; SPSS) statistical software packages were used for the analyses.

3 Abbreviations used in this paper: gpe, Gag-Pol-Env; ICC, intracellular cytokine staining; LPR, lymphoproliferative response; rtn, Retanef.
Results
Addition of immunogens encoded by early regulatory genes decreases the level of acute viremia and increases protection from simian AIDS

To assess the effect of immunization with early regulatory proteins, we have generated plasmid DNA and NYVAC-based vaccines expressing a chimeric polypeptide Retanef comprising genetically modified, attenuated, and reassorted SIV Rev, Tat, and Nef (DNA-rtn and NYVAC-SIV-rtn) (55) and directly assessed the effect of immunization with the chimeric Retanef vaccine alone or in combination with the Gag-Pol-Env (gpe) vaccine (8, 9). Twelve animals in the control groups were either not immunized (group 1A, four animals) or immunized with mock NYVAC (group 1B, eight animals; Fig. 1). DNA immunizations were given at weeks 0, 4, and 12 to animals in groups 2, 3, and 4; boosts with NYVAC-SIV vaccine were given at weeks 24 and 52 (Fig. 1). Animals in group 2 were immunized with the Retanef vaccine, animals in group 3 received both Retanef and gpe vaccines, and animals in group 4 received only the gpe vaccine. All animals were exposed to intrarectal challenge with 30 mucosal infectious doses of SIVmac251 virus during the memory phase of the immune response (6 mo after the last immunization). The immunization regimen of DNA plus recombinant NYVAC-SIV stock and the challenge virus used in all groups were the same to allow for a direct comparison. The results of the challenge of animals in groups 1B and 4 were described previously (9) and demonstrated that immunization with Gag, Pol, and Env provided a significant virological benefit during acute and chronic infection (Fig. 2A).

After intrarectal challenge exposure with SIVmac251, the animals immunized with Retanef alone (group 2) displayed a slight delay in the onset of blood viremia (Fig. 2, B–D) and a statistically significant decrease in acute viremia compared with control animals (p = 0.023; Fig. 3A and Table I). Two of the macaques immunized with Retanef alone controlled viremia below 10^4 copies/ml plasma and maintained normal levels of CD4+ T cell counts, whereas the remaining six animals experienced high viremia and died of AIDS within 32 mo (Fig. 2B, middle panel).

Importantly, immunization with Retanef in conjunction with gpe (group 3) resulted in a 3- to 7-day delay in the onset of viremia (Fig. 2, B and C) and a marked decrease in acute viremia compared with controls (p < 0.001), group 2 (p < 0.001), or group 4 (p = 0.028). Five of eight animals in this group contained virus below the level of detection or displayed a 2 log_{10} reduction of peak acute viremia. In two animals (no. 823 and 3165), virus was mostly contained at <500 RNA copies/ml plasma during the acute and chronic phases of infection (Fig. 2B, lower panel). These animals, however, were infected, because SIV DNA was detected in PBMCs (data not shown), and occasional “blips” of viremia were observed (animal 3165 at week 53 and animal 823 at week 70; Fig. 2B, lower panel). The peak of viremia in the remaining three animals appeared sharper and declined earlier than in controls.

The level of chronic viremia in group 3 was significantly lower than that in the controls (p = 0.004). Following the set point of viremia, five of eight immunized animals in group 3 maintained plasma levels at <10^4 copies/ml, whereas only one of 12 control animals did so (Fig. 3B). Four of eight animals in group 3 remained healthy 32 mo after challenge exposure (Fig. 3C). Three of them (animals 692, 823, and 3165) maintained normal CD4+ T cell counts and low virus levels, whereas animal 822 experienced a progressive decline in CD4+ T cells (data not shown).

Collectively, the results demonstrate that the addition of early regulatory genes to the structural genes in the vaccine resulted in a delay and significant reduction of acute viremia. The difference between groups 3 and 4 in their abilities to control virus during the chronic phase of infection did not reach statistical significance; however, these data are probably skewed by the presence of a higher number of Mamu-A*01-positive animals. Immunization with SIV-gpe

Evidence of Ag competition after vaccination and challenge with SIVmac251

The design of these studies provided the opportunity to assess whether combining multiple Ags would result in Ag competition. At first we focused on the dominant epitope Gag_CM9 recognized by Mamu-A*01-positive animals. Immunization with SIV-gpe...
Ags induced higher responses in animals in group 4 than in macaques immunized with the combination of SIV-gpe and SIV-rtn vaccines (group 3; Fig. 4A). Similarly, after the first boost with recombinant NYVAC, animals immunized with SIV-gpe alone displayed Gag- and Env-specific proliferative responses of higher peak magnitudes than those observed in animals vaccinated with SIV-gpe together with SIV-rtn (Fig. 4B). Consistent with these data, proliferative responses to Nef Ag were higher in macaques immunized with Retanef only than in those immunized with multigenic vaccines after the first recombinant NYVAC boost ($p = 0.007$; Fig. 4B). These intergroup differences were not evident after the second NYVAC boost, with the exception of Tat lymphoproliferative responses (LPRs), which were higher in group 2 than in group 3 ($p = 0.05$; weeks 53–56). Collectively, these data suggest that the combination of several immunogens decreases the relative frequency of specific T cells for a given Ag. This difference was evident and reached statistical significance at the time of maximal expansion of the immune response induced by the vaccines.

After mucosal exposure to SIVmac251, all animals became infected. Mamu-A*01-positive macaques; †, animal was euthanized due to severe health problems. C, Mean plasma levels of viral RNA in individual groups. Error bars indicate the SEs.
Consistent with the finding of epitope competition, macaques immunized with SIV-rtn alone experienced a faster and larger expansion of Tat_SL8-specific CD8+ T cells (Fig. 5A, day 21) than either control macaques or macaques immunized with SIV-gpe. Likewise, animals immunized with gpe only had higher frequency and faster kinetics of appearance of Gag-specific cells (day 13) than either controls or animals immunized with Retanef. A balanced and more muted anamnestic response to these Ags was observed in animals receiving both the gpe and Retanef vaccines (Fig. 5A). The data confirm and add support to the observation of Ag competition during both primary and anamnestic responses.

LPRs to Gag p27 in the first 2 mo of infection were significantly higher in SIV-gpe-immunized groups 3 and 4 than in controls (p < 0.001 and 0.007, respectively) or the SIV-rtn only-immunized group 2 (p = 0.002 and 0.02), suggesting that vaccination had primed these responses (Fig. 5B). Similarly, Tat LPRs were higher in groups 2 and 3 than in control group 1 (p = 0.004 for both; Fig. 5C) and were preserved better in macaques immunized with the vaccine combination.

Next, SIV-specific CD4+ and CD8+ T cell anamnestic responses in the infected macaques were determined by ICC of cells stimulated with pools of overlapping 15-meric peptides. Macaques immunized with gpe (groups 3 and 4) had significantly higher numbers of Gag-specific CD4+ T cells (p < 0.03) and a trend for higher CD8+ T cells compared with other groups, whereas macaques immunized with Retanef only displayed a trend for an increase in the Rev, Tat, and Nef responses (Fig. 5D). The responses in group 3 were balanced, without apparent polarization toward any Ag.

Gag-specific CD4+ T cell responses after infection were higher in gpe-immunized groups 3 and 4 compared with controls or group 2, demonstrating a classical recall response to Gag (Fig. 5B). Similarly, the frequencies of Rev-, Tat-, and Nef-specific CD4+ T cells
were higher in animals immunized with the Retanef only, suggesting an anamnestic response to early regulatory genes (Fig. 5D).

The anamnestic responses with all these specificities were more variable in macaques from group 3 that had been immunized with a combination of gpe and Retanef vaccines.

Virological and immunological correlates of long-term containment of viral replication

The availability of virological and immunological data on a total of 24 immunized animals and 12 control animals provided the opportunity to assess the relationship between early and late virus levels as well as how the immune response induced by vaccination affected the virological outcome. We observed a direct correlation between the mean plasma virus RNA levels during primary infection and the mean plasma virus level after the set point (Fig. 6A), providing support for the concept that T cell-based vaccines that curtail early in infection are able to alter viral replication and delay the disease course. We have previously shown that preservation of Gag-specific CD4+ T cells is important in determining the long-term virological outcome (8, 9, 63). We therefore analyzed a total

### Table II. MHC haplotypes of rhesus macaques used in the study

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* Values of p are based on two-tailed Mann-Whitney rank sum test. Acute viremia is defined as the average viremia at days 13, 20, and 28; chronic viremia is the average viremia at weeks 16, 20, and 24 postchallenge.
of 29 macaques for the frequency of Gag-specific CD4$^+$ T cells by ICC within a few weeks after infection and confirmed an inverse correlation with both acute and post-set point viremia levels ($p < 0.001$; Fig. 6B).

Confirming this observation, the LPRs to Gag Ag throughout the first 2 mo of infection also inversely correlated with both acute and post-set point viremia (Fig. 6C); LPR to Tat correlated with acute ($p = 0.01$), but not post-set point viremia (data not shown). In contrast, the inverse correlation between the numbers of Gag-specific CD8$^+$ T cells and acute or chronic viremia was weak and reached significance only on day 20 (Fig. 6D), but not on day 13 or at any other time after challenge. Together, these data suggest that the reduction of primary viremia results in a better preservation of Gag-specific CD4$^+$ T cell responses, which, in turn, may contribute to the maintenance of CD8$^+$ effector T cell function.

**Discussion**

Previously, we have demonstrated that immunization with SIV gpe in a DNA prime/poxvirus boost regimen confers significant suppression of viremia after mucosal challenge with highly pathogenic SIV$_{mac251}$ (9). This study provides direct evidence of the benefit of addition of early regulatory proteins, Rev, Tat, and Nef, to the vaccine regimen. Immunization with a combination of early and structural/enzymatic proteins resulted in a 3- to 7-day delay in
acute infection, significant reduction of viral load in the initial phase of infection, lower set point virus levels, and increased survival. In contrast, vaccine based on regulatory SIV genes alone conferred only a slight delay and less significant reduction of acute infection and had little effect on the overall course of disease.

During the chronic phase of infection, virus levels did not differ significantly among the animals immunized with all Ags and those immunized only with gpe. We believe that this may be due to the presence of more animals in group 4 expressing Mamu-A*01 MHC allele (five of eight animals) compared with group 3 (two of eight), because the Mamu-A*01 molecule has been associated with natural resistance to SIV replication (58, 59). The underlying effect of given MHC class I haplotypes on disease progression was also addressed by screening macaque DNA for the eight most frequent Mamu alleles (Table II). With the exception of Mamu-A*01, no association between any particular Mamu allele and viral load was found.

The added beneficial effect of immunization with early proteins may be explained either by broadening immune responses or, alternatively, by special qualitative properties that distinguish these proteins from other viral Ags. CTL recognition of Nef, Rev, and Tat epitopes on cells in the early stage of infection results in their elimination before progeny virus is released (17, 21). In a series of elegant experiments, van Baalen et al. (22) have shown that CTL recognition of early, rather than late, Ags resulted in a 2- to 3-log reduction of virus production by similar numbers of effector cells in 10 days of culture. This qualitative difference between CTLs with specificity toward early vs late proteins may be especially important in the initial phase of infection in the absence of neutralizing Abs to Env (64) and could account for the delay of detectable virus in Retanef-immunized groups 2 and 3. Similar reduction and/or delay of acute viremia were observed in previous studies assessing the effect of immunization with early genes (20, 34, 43, 65). It should be noted that the level of the chimeric Retanef Ag used in this study was relatively low, at least as evidenced by the low levels of CD8 T cells induced against the immunodominant Tat_SL8 epitopes during vaccination, which contrast with the high levels of this response after infection. Thus, the

FIGURE 5. Immune responses after the challenge exposure to SIVmac251. A, Postchallenge T cell responses to Gag_CM9 and Tat_SL8 epitopes as measured by tetramer staining of PBMCs in all groups. Error bars indicate the SEs. B and C, Postchallenge LPRs to Gag p27 and Tat proteins. Error bars indicate the SEs. D, Relative percentages of blood T cells specific for individual SIV Ags detected by ICC staining assay on days 0, 13, 20, and 42 after challenge with SIVmac251. Group mean percentages of ICC cattle cells of total CD3 CD8 or CD3 CD8 (CD4) cells are shown; error bars represent the SEs.
beneficial effect of immunization with early viral proteins may be enhanced by using improved immunogens.

In this study we demonstrate that the reduction of the extent of acute SIV viremia has important implications for disease progression, because the level of acute viremia predicts the long-term course of infection (Fig. 6A). This is in line with previous observations (66). It has recently been demonstrated that a massive initial infection of memory CD4\(^+\) T cells results in a depletion of 30–90% of these cells from the lymphoid tissues and mucosal surface during the first 2 wk of infection (1–3). The early destruction of the CD4\(^+\) memory compartment represents an initial insult from which the organism may never recover and which may be the distinguishing feature setting HIV apart from chronic infections with other nonlytic viruses (5). The loss of CD4\(^+\) T cell regulatory and effector functions, especially at the tissue-environmental interfaces, may increase the vulnerability to pathogens, contribute to chronic immune hyperactivation, and contribute to the destruction of lymphoid tissue. Our results support the idea that rapid recruitment of pre-existing vaccine-induced T cells specific for early Ags may be important in the

**FIGURE 6.** Virological and immunological correlates. Inverse correlation between the levels of acute (days 13–28) and post-set point (weeks 16–24) viremia in all 36 macaques studied (A). The \( p \) value was determined by Spearman rank-order correlation of data in 36 animals. *, Overlapping data from three animals. B, Correlation between the levels of acute (days 13–28) and post-set point (weeks 16–24) viremia and Gag-specific CD4\(^+\) T cell responses detected on day 13 after challenge. C, Average Gag-specific LPRs (stimulation index) detected at weeks 2–8 after infection. D, Gag-specific CD8\(^+\) T cell responses on day 20 after challenge. Correlation coefficients (\( R \)) and \( p \) values were determined by Spearman rank-order correlation test. Data from 29 of the 36 animals are presented.
initial suppression of virus growth and preservation of memory CD4+ T cell function.

There are several lines of evidence that support this hypothesis. HIV-specific CD4+ T cells that maintain proliferative capacity are detected in long-term nonprogressors and patients treated with highly active antiretroviral therapy; however, they are drastically reduced in number and/or function in individuals with high levels of plasma HIV (67, 68). These cells were shown to be a preferential target of HIV (69), an observation not surprising given their spatial and temporal colocalizations with HIV-producing cells in the local microenvironment of infected lymphoid tissue. We and others (63, 67, 70–72) have demonstrated that early suppression of acute viremia with antiretrovirals results in a preservation of virus-specific CD4+ T cell responses and long-term control of infection. In this study we confirm and extend our previous observation that the ability to control infection directly correlates with the preservation of Gag-specific CD4+ T cells and their proliferative capacity during primary viremia (Fig. 6, B and C). These data go hand-in-hand with the idea that early expansion of vaccine-induced virus-specific T cells may result in the preservation of pre-existing and/or newly recruited virus-specific CD4+ T cells. Although the immunization with early proteins alone delayed the onset of viremia, the benefit was rapidly lost, probably due to virus escape from immune recognition (23). Accumulated evidence suggests that more stable epitopes, such that the virus cannot escape without a significant toll on its fitness, are needed for long-term maintenance of protection (73–75). This could explain our observation that a combination of early and structural genes improved the overall control of viral replication.

The data presented in this study are germane to the ongoing debate on whether to include early viral proteins as part of an HIV vaccine. Among the arguments used against the inclusion of these proteins in a vaccine is that their sequence is highly variable at each amino acid position, and only a few conserved regions can be identified. A second argument stems from the fact that presentation of these early Ags in the acute phase of infection may focus the immune response toward epitopes that can readily escape without affecting viral fitness. Thus, early recognition of these epitopes may divert responses to more conserved and protective epitopes, the mutation of which affects viral fitness (72, 73). Our data suggest that an effective HIV vaccine should induce broad responses to both types of epitopes. Despite their transient character, the responses directed to the epitopes that are subject to variation without significant loss of viral fitness, such as those found in early regulatory proteins, may still wield a significant effect on the control of virus proliferation during the decisive initial days of infection. The Retanef Ag used in this study constitutes only 16% of the protein sequence of all Ags used in the complex vaccine, suggesting that the delay and lower level of acute viremia may be due to qualitative differences in immune responses to the respective Ags.

An important aspect of this study is the observation of Ag competition, manifested by a relative reduction of CD8+ T cell responses to Gag and Tat and LPRs to Gag, Env, Tat, and Nef after immunization with combined vaccines. This reduction probably results from competition for a common niche and reflects possible size limits of effector CD8+ and CD4+ T cell populations (76–78). The competition is probably accentuated by a concomitant induction of T cells specific for the vaccinia virus backbone (79). The dominance of secondary virus-specific responses after the second boost with recombinant NYVAC vectors could explain the lower overall responses to SIV Ags as well as the apparently lesser competition between them. Possibly, staggering immunization temporally and spatially with DNA and recombinant poxviruses expressing diverse Ags will improve vaccine immunogenicity and relative efficacy.

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Disclosures
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