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Long-Term Control of Simian Immunodeficiency Virus\textsubscript{mac251} Viremia to Undetectable Levels in Half of Infected Female Rhesus Macaques Nasally Vaccinated with Simian Immunodeficiency Virus DNA/Recombinant Modified Vaccinia Virus Ankara

Mariana Manrique,*,† Pamela A. Kozlowski,‡ Antonio Cobo-Molinos,*,† Shainn-Wei Wang,*,†,¹ Robert L. Wilson,‡ David C. Montefiori,§ Keith G. Mansfield,¶ and Anna Aldovini*¹

The efficacy of two SIV DNA plus recombinant modified vaccinia virus Ankara nasal vaccine regimens, one combined with plasmids expressing IL-2 and IL-15, the other with plasmids expressing GM-CSF, IL-12, and TNF-α, which may better stimulate humoral responses, was evaluated in two female rhesus macaque groups. Vaccination stimulated significant SIV-specific mucosal and systemic cell-mediated immunity in both groups, whereas SIV-specific IgA titers were sporadic and IgG titers negative. All vaccinated animals, except one, became infected after intravaginal SIV\textsubscript{mac251} low-dose challenge. Half of the vaccinated, infected animals (7/13) promptly controlled virus replication to undetectable viremia for the duration of the trial (130 wk) and displayed virological and immunological phenotypes similar to those of exposed, uninfected individuals. When all vaccinated animals were considered, a 3-log viremia reduction was observed, compared with controls. The excellent viral replication containment achieved in vaccinated animals translated into significant preservation of circulating CD4+ T cells and in circulating and mucosal CD4+/CD8+ T cells and in reduced immune activation. A more significant long-term survival was also observed in these animals. Median survival was 72 wk for the control group, whereas >50% of the vaccinated animals were still disease free 130 wk postchallenge, when the trial was closed. There was a statistically significant correlation between levels of CD4+/IFN-γ+ and CD8+/IFN-γ+ T cell percentages on the day of challenge and the control of viremia at week 60 postchallenge or survival. Postchallenge immunological correlates of protection were systemic anti-SIV Gag + Env CD4+/IL-2+, CD8+/IFN-γ+, and CD8+/TNF-α+ T cells and vaginal anti-SIV Gag + Env CD8+ T cell total monofunctional responses. The Journal of Immunology, 2011, 186: 000–000.

Recent efforts toward the development of an AIDS preventive vaccine have focused mainly on vaccines aimed at stimulating T cell-mediated immunity, in part because these vaccines had shown very promising results in preclinical studies in macaques challenged with simian HIV (references in Refs. 1, 2) (3–7). However, these approaches were less successful in protection against SIV challenge (2). In this model, a substantially smaller reduction of the viremia and only a temporal protection from CD4+ T cell loss that lasted ∼6–8 mo after challenge with SIV was observed (8, 9). The DNA/live vector approach predominantly simulates T cell-mediated immunity, with poor stimulation of systemic and mucosal humoral immunity (8–10). Moreover, an HIV-expressing adenovector approach tested in humans failed to provide protection from infection and to reduce viral loads in infected individuals, and increased HIV-1 infection rates were observed in subgroups of vaccine recipients compared with controls (11, 12). However, recent results of vaccination in humans with four doses of inactivated HIV recombinant pox virus plus two injections of an HIV rgp120 showed that the vaccine efficacy in preventing infection was ∼30%. This vaccine had low levels of T cell immunogenicity (∼20%) and did not provide viremia control or CD4+ T cell protection in the vaccinees that contracted HIV during the trial (13).

Data from mucosal immunization in humans suggest that IgA responses are maximal at the site of mucosal exposure and present, albeit to a lesser degree, at other mucosal sites (14–16). Rectal immunization works best to achieve high IgA titers in rectal secretions, whereas responses are not optimal in vaginal secretions. Similarly, vaginal immunization provides the best responses for vaginal immunity. Nasal immunization can elicit rectal as well as vaginal responses, with better vaginal responses than those elicited by rectal immunization (16). During mucosal immunization, systemic responses can also be primed, although not as efficiently as with systemic routes. If immunity is desired at more than one site, some mucosal sites appear to be better suited than others, and the nasal route may be best for this goal (15).
Our interest in investigating the nasal mucosal route in female animals stemmed from a number of considerations. The nasal route is particularly attractive because of its simplicity and proven efficacy (16–18). Significant mucosal and systemic responses might be achieved after nasal immunization in primates with an appropriate adjuvant and boosting regimen. In mice, levels of serum IgG Ab and splenic CTLs induced after nasal immunization were reported to be comparable to those generated after i.m. or i.p. injection. After nasal immunization, CTLs were induced in lymph nodes draining respiratory and female genital tract mucosa, whereas in parenteral immunization they were not (19, 20). Nasal immunization in mice and monkeys also induced migration of Ag-specific B lymphocytes to many mucosal tissues, judging by the appearance of IgA Ab in secretions of the salivary glands, upper and lower respiratory tracts, intestine, and female genital tract (17, 18, 21). These results have also been confirmed in humans (16, 22, 23). Increasing evidence in HIV-infected individuals suggests that the sexes progress differently to AIDS, mainly because of differences in the control of HIV infection and HIV-induced immune activation (24–26). As these differences may also translate into differences of vaccine efficacy postinfection, and given that almost half of the worldwide HIV-infected population are women, it is important to evaluate the efficiency of vaccine candidates in both sexes.

Although the results obtained thus far with SIV DNA vaccination in animal models are promising, there is clearly a need to increase the potency of this approach. The magnitude of the Ag-specific response can be modulated with the addition of genes for certain cytokines (10, 27–29). The type and extent of modulation depend upon the cytokine used and the route of vaccination. In general, administration of Th1 cytokine genes led to increased cell-mediated responses, whereas Th2-type cytokines mainly increased humoral responses (10, 30). Given that mucosal responses, in addition to systemic responses, might be necessary for protection against sexual transmission of pathogens like HIV, it would be useful to identify cytokines that enhance mucosal immunization in macaques. IL-2 is known to broadly stimulate immune responses, and IL-15 affects the longevity of Ag-specific immune responses in mice (29, 31–33). The ability of IL-12 to stimulate Ag-specific responses systemically and mucosally supports it as an ideal adjuvant cytokine for an SIV vaccine (28, 32). GM-CSF increases different Ag-specific responses, probably via a mechanism that affects dendritic cell Ag presentation (27, 31, 34). IL-12 and GM-CSF may better stimulate B cell activity, and in particular IgA production, either by being a growth factor for B cells (IL-12) or by affecting the Ag presentation activity of dendritic cells with subsequent expansion and differentiation of B and T cells (GM-CSF) (27, 29, 31, 34, 35). In mice, the GM-CSF/TNF-α/IL-12 cytokine combination showed efficacy in increasing the immunogenicity of HIV and other Ags after s.c. or rectal vaccination (29, 35).

In this study, two vaccine regimens administered via the nasal route were evaluated in female rhesus macaques, one being a vaccine previously tested in male rhesus macaques (3, 4, 7) and the other including as adjuvants TNF-α, IL-12, and GM-CSF DNAs. The goals were 1) to evaluate which of the two nasal SIV DNA recombinant modified vaccinia virus Ankara (rMVa) prime/boost regimens provided the broadest, qualitatively more diverse immune responses, as well as the level of protection from SIV infection and progression to AIDS; and 2) to investigate immunological correlates of protection.

Materials and Methods

Vaccine constructs

The DNA plasmid pVacc6 used in the vaccination is a derivative of pVacc1 (36). pVacc6 includes a full SIVmac239 genome with multiple mutations in the NC basic domain and the functional domains of RT, INT, and PR and a stop codon at the beginning of the vpr gene. Gene expression is under the control of the CMV promoter, with deletions of both long terminal repeats. The DNA sequence was confirmed by sequencing, and the profile of the viral particle produced was evaluated as previously described (36). rMVa expressing SIV Gag, Pol, and Env proteins was prepared as previously described (37). The IL-2/IG and IL-15, TNF-α, IL-12, and GM-CSF plasmids were previously described (29, 30). IL-2/IG, IL-15, TNF-α, IL-12, and GM-CSF production from these plasmids was tested in the supernatants of transfected 293T by ELISA.

Experimental groups and vaccination schedule

Female rhesus macaques were cared for at the New England Regional Primate Research Center, using approved protocol under the guidelines established by the Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Fourteen animals were divided into two groups that received intranasal immunizations: 1) the DNA vaccine; a saline solution (Sigma-Aldrich, St. Louis, MO) was used to resuspend the DNA, and the concentration was adjusted to 10 mg/ml. The mucosal administration was as follows: group 1 (n = 7), each DNA immunization consisted of 2.5 mg pVacc6, 0.5 mg IL-2/IG, and 0.5 mg IL-15; and group 2 (n = 7), 2.5 mg pVacc6, 0.5 mg GM-CSF, 0.5 mg IL-12, and 0.5 mg TNF-α, administered as 350 μl or 400 μl, half in each nostril of the animal. These three DNA doses were administered on days 0, 1, week 9, and week 25. A total of 10^9 PFU of rMVa, expressing SIV gag, pol, and env genes, were delivered intranasally at week 33. Starting from week 60, each animal was inoculated with a low dose (~0.2 doses of a 50% animal infectious dose evaluated in a rhesus macaque vaginal titration and corresponding to 100 50% tissue culture infective doses) of the pathogenic SIVmac251 virus grown in rhesus macaque PBMCs (a gift from Dr. Nancy Miller, National Institutes of Health, National Institute of Allergic Diseases, Bethesda, MD, and Dr. R. Desrosiers, New England Primate Research Center, Southborough, MA), administered nontraumatically with needleless tuberculin syringes as cell-free virus in the vagina. In parallel, six naive animals (control group) were similarly inoculated with a low dose. This virus stock is a highly diverse swarm that contains many different virus species and quasispecies.CCR5 viruses (38). Doses were repeated weekly until an RT-PCR assay on plasma-derived virus yielded a positive result. One animal per group was Mamu-A*01+ (no. 317, no. 136, no. 8). Other animals with protective alleles were no. 381 (Mamu-B*17), no. 270 (both Mamu-B*08 and Mamu-B*17), and no. 451 (Mamu-B*08).

Analysis of SIV-specific IgA in rectal and vaginal secretions and IgG in serum

Rectal and vaginal secretions were collected before and after immunization, as described previously (39). Antiviral and total IgA in rectal and vaginal secretions and IgG in serum were measured by ELISA, as described (4, 7). Briefly, for the ELISAs, microtiter plates were coated overnight with 100 ng/well SIVmac251 gp130 (ImmunoDiagnostics, Woburn, MA) or 100 μl well of 1/400 SIV viral lysate (Advanced Biotechnologies, Columbia, MD) that lacks detectable envelope protein at this dilution. Reactivity detected against the SIV lysate is therefore described as being against SIV Gag and Pol proteins. For SIV IgA assays, plasma from previously vaccinated macaques that developed gp130- or Gag- and Pol-specific IgA after challenge was depleted of IgG using Protein G Sepharose (GE Healthcare, Piscataway, NJ) and used as standards. For SIV IgG assays, the standard was pooled plasma from SIV-infected macaques. All SIV standards had been calibrated relative to the total IgA or IgG standard by coating portions of the same plate with SIV Ag, goat anti-monkey IgA (Alpha Diagnostics, San Antonio, TX), or goat anti-monkey IgG (MP Biomedical). Bound IgG was detected with Protein G Sepharose (GE Healthcare, Birmingham, AL) or -monkey IgG (Alpha Diagnostics). Secretions had to satisfy two criteria to be considered IgA Ab positive: 1) the specific activity (SA); nanograms of anti-SIV IgA per microgram total IgA to gp130 or SIV lysate had to be ≥0.145 or 0.224, respectively, which represents the mean SA + 3 SD for rectal secretions of naive macaques; and 2) the fold increase (immunization/preimmunization) in SA had to be ≥3.4-fold. If a preimmunization secretion had no detectable IgA Ab, it was assigned the mean SA value of naive macaques (0.049 for gp130; 0.083 for SIV lysate). SIV Gag- and Pol-specific plasma IgG was measured by ELISA using plates coated with SIV lysate as described above. To obtain titers of SIV Gag- and Pol-specific IgG in plasma, the Ab concentrations (in micrograms per milliliter) were multiplied by 1600, based on the finding of previous studies that this is the average of endpoint titer for 1 μl/ml IgG Ab in this assay (3, 4). Neutralization titers were measured as a function of Tat-induced luciferase reporter gene expression after single round of infection in TZM-bl cells (3). The virus
used in the neutralization assay was SIVmac251. Titters of SIV-specific neutralizing Abs are the serum dilution at which relative luminescence units were reduced 50% compared with virus control wells after subtraction of background. The assay stock of primary SIVmac251 was prepared in human PBMCs and titrated in TZM-bl cells.

### Isolation of intestinal mononuclear cells and PBMCs

After Telazol anesthesia, seven or eight biopsies per animal per time point were obtained from the rectum, using sterile forceps and a small pinch biopsy device (Olympus endoscopic biopsy forceps). Mononuclear cells (MNCs) from colon-rectal biopsies and PBMCs were isolated according to previously published procedures (40).

### Intracellular staining and Abs

MNCs (10^5 cells) and PBMCs (10^6) were incubated for 14 h with medium alone (unstimulated) or 1 μg/ml pool of 15-mer SIV Gag or SIV Env peptides. As a positive control, cells were incubated with 10 ng/ml PMA (Sigma-Aldrich) and 1 μg/ml ionomycin (Sigma-Aldrich); background controls were provided by unstimulated cells. All cultures contained Brefeldin A (BD GolgiPlug, cat. no. 555029; BD Biosciences) and 1 mg/ml anti-CD49d and anti-CD28. The PBMCs and MNCs were stained and evaluated for expression of cytokines according to previously described procedures and reagents (4). Two additional Abs, AT-1 (CD38) and Act-1 (α-S7) (41), were also used in this study. For MNCs, 200 ml viability dye (VIVID, LIVE/DEAD kit, Invitrogen) was added to the Ab mixture to exclude dead cell background. The CD3+ cells were used as the gate formultiplicative expression profiles were determined using the Boolean gating function of FlowJo software. We define as monofunctional those cells producing upon stimulation only one of the cytokines tested (IL-2, IFN-γ, TNF-α) and as multifunctional those cells producing upon stimulation simultaneously two or three of the cytokines tested.

### Viral load quantitation

Plasma SIV RNA levels were measured by real-time RT-PCR assay, as described (42). The assay has a threshold sensitivity of 30 copy-equivalents per milliliter. Interassay variation is <25% (coefficient of variation).

### Statistical analysis

Calculations and statistical analyses were performed using the GraphPad Prism version 3 software. Endpoint Ab titers and RNA viral loads were logarithmically transformed, and the geometric means were calculated for each vaccination group. The two-tailed Fisher’s exact test was used to compare the frequency of rectal IgA responses between groups. Between-group comparisons were carried out by two-tailed t test or Mann–Whitney U test. Correlation analysis was done using the Spearman rank test. Survival distributions were evaluated using the Kaplan–Meier method, and the log-rank test was used to compare survival curves between groups. Results of statistical analyses were considered significant if they produced p values ≤0.05.

### Results

#### Virus-specific humoral and T cell responses during immunization

Toward the goal of achieving significant humoral responses, we compared in female rhesus macaques the immunomodulatory

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**Table I. SIV-specific Abs in prechallenge secretions**

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**Fold Increase of SIV Env or SIV Gag and Pol SA for IgA in Rectal Secretions**

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Postimmune/preimmune fold increases in IgA SA measured by ELISA against SIV gp130 Env or SIV lysate Gag and Pol proteins are shown before and after the slash, respectively. To be significant, the SA had to be greater than the mean SA + 3 SD for native secretions and 3.4-fold above the animal’s preimmune SA. Only significant increases are presented.

—, not significant; SA, nanograms of IgA Ab per microgram of total IgA.
activity of the GM-CSF, TNF-α, and IL-12 DNA combination in conjunction with the SIV viral vector pVacc6 with that of the IL-2/IL-15/SIV DNA regimen previously tested in male animals (7). The IL-2/IL-15 combination is known to broadly stimulate cell-mediated immune responses and affect their longevity in mice (29, 31–33); the other should stimulate immune responses in general and humoral responses in particular (28, 32). Two groups of seven female rhesus macaques were nasally primed with three doses of SIV/cytokine DNAs at weeks 1, 9, and 25 and boosted with one dose of MVA expressing SIV Gag, Pol, and Env proteins at week 33. Group 1 received the IL-2/IL-15 DNA adjuvant combination; group 2 received a mix of IL-12, GM-CSF, and TNF-α DNAs.

Humoral and cell-mediated virus-specific immune responses were measured in PBMCs and in mucosal tissues (colorectal and cervicovaginal biopsies) during immunization. Neither adjuvant cytokine combination was found to induce SIV Env-specific IgG in plasma, although macaques 129 and 111 demonstrated 4.8- and 6.8-fold increases of SIV Gag- and Pol-specific IgG, respectively, at week 36 (not shown). Rectal and vaginal secretions collected at monthly intervals after vaccination infrequently contained significant levels of anti-SIV IgA Ab, especially against Env, at more than one time point during the immunization phase of the study (Table I). Macaque 111, who remained uninfected after 35 vaginal challenges, persistently demonstrated anti-Gag, Pol IgA in rectal secretions, but no antiviral IgA in vaginal secretions (Table I). The only vaginal IgA detected just before challenge was against Gag, Pol (Table I). These were present in animals 381, 197, and 136, all of which became infected but rapidly controlled viremia. Overall, these results are consistent with our previous observations that nasal immunization of male macaques with this DNA/rMVA vaccine infrequently induces SIV-specific systemic or mucosal Ab responses. They further suggest that neither of the adjuvant cyto-

**FIGURE 1.** SIV Gag + Env-specific cell-mediated immune responses during immunization. A. Group means of number of CD3+/CD4+ or CD3+/CD8+ SIV-specific T cell-producing IL-2, IFN-γ, and TNF-α of 10⁶ PBMCs after stimulation with SIV Gag or Env peptide pool were calculated for each vaccinated group (group 1, blue; group 2, red). Error bars represent SEM. B and C, SIV-specific cell-mediated immune responses in rectal and vaginal mucosa MNCs during immunization. Numbers of SIV Gag + Env-specific CD4+ and CD8+ T cells of 10⁶ total cells detected in MNCs isolated from rectal biopsies (B) or vaginal biopsies (C) at week 36 (represented in blue or purple shades) and 40 (represented in brown or teal shades) since the beginning of the immunization are shown for each animal. These time points correspond to 4 and 8 wk after rMVA boost. **Left panels** show the percentage of cells producing IL-2 or IFN-γ or TNF-α upon stimulation with SIV Gag and Env peptide pools (monofunctional responses), and **right panels** illustrate percentages of cells simultaneously producing IFN-γ/TNF-α or IFN-γ/IL-2 or IL-2/TNF-α (multifunctional responses).
Circulating T cell responses were measured using flow cytometry by evaluating the number of T cells producing IL-2, IFN-γ, and TNF-α in PBMCs after stimulation with SIV peptide pools. In both vaccinated groups, SIV-specific T cell responses could be detected in PBMCs, and no significant differences were observed between the two (Fig. 1A). A higher frequency of rectally derived SIV-specific total monofunctional CD4+ or CD8+ T cells were observed in both vaccinated groups (Fig. 1A, right panels). These responses returned to baseline levels by the time of challenge.

Significant differences in SIV-specific T cell responses were observed in mucosal compartments between groups after rMVA boosting. A higher frequency of rectally derived SIV-specific plus SIV Env-specific CD4+/IL-2+ and CD4+/IFN-γ+ T cells could be detected at weeks 12 and 28 after the second and third DNA dose. These responses did not differ between groups (Fig. 1A). This was also true for CD8+ T cell responses post-immunization (Fig. 1A). Lower levels of SIV-specific multifunctional CD4+ or CD8+ T cells were observed in both vaccinated groups (Fig. 1A, right panels). These responses returned to baseline levels by the time of challenge.

FIGURE 2. A. Plasma viral loads: logarithmic mean values for plasma viral load for vaccinated (black) and control (green) animals (left panel), for each individual group (middle panel). Values for each animal are reported in the right panel. Error bars represent SEM. Peripheral blood CD4+ T cell absolute counts and percentages postinfection. B, Mean CD3+/CD4+ T cell absolute counts per microliter, percentage of CD3+/CD4+ T cells, and Kaplan–Meier survival curves for the control group and the animal group that includes all the vaccinated animals. C, Mean CD3+/CD4+ T cell absolute counts per microliter, percentage of CD3+/CD4+ T cells, and Kaplan–Meier survival curves for the control group and each vaccinated group. Error bars represent SEM. The number reported in the Kaplan–Meier survival curves indicates the number of animals that died of AIDS of the total number of animals in the group.
point and during the infection chronic phase. Seven animals (three of group 1 and four of group 2) controlled viremia to undetectable levels after a virus peak was detected in the blood at week 2 or 4. At least two positive time points were detected in each of these animals. They maintained this control up to week 60 postinfection, when the last viral load measurement was obtained (Fig. 2 right panel), and they were still RT-PCR negative on the day of euthanasia (week 130 postinfection). These results indicate that the pre-existing antiviral immunity stimulated by the vaccination permitted a more efficient control of viral replication postinfection than did the immunity developed by the vaccination-naive animals during the SIV infection.

**Circulating CD4+ T cell analysis and progression to AIDS**

The analysis of circulating CD4+ T cell population dynamics indicated that, as expected based on the RNA viral loads, vaccinated
macaques maintained higher levels of CD4+ T cell counts (Fig. 2B, 2C) than did control animals during the course of the infection. Significant differences were observed between the CD4+ T cell absolute count average of all vaccinated animals and controls (p = 0.02, Fig. 2B, left panel), between group 2 and controls (p = 0.0001), and between group 2 and group 1 (p = 0.002, Fig. 2C, left panel) (Mann–Whitney U test, two-tailed p value). When CD4+ T cell percentage averages measured from week 20 to week 52 postinfection were compared between vaccinated animals and the control group, vaccinated animals maintained higher CD4+ T cell percentages than did controls (p = 0.0079, Fig. 2B, middle panel). This was also true when the comparison was done between group 2 and group 1 (p = 0.008) and group 2 and controls (p = 0.008) (Fig. 2C, middle panel).

Long-term protection from disease and survival, measured as time to diagnosis of AIDS and euthanasia, was evaluated in vaccinated and control groups and reported in the Kaplan–Meier curves in Fig. 2B and 2C. At week 130, the trial was ended and the surviving animals were euthanized even if healthy. Longer median survival was observed in each immunized group (>130 wk), compared with the naïve group (median survival of 62 wk, p = 0.01, Mantel–Cox log-rank test) (Fig. 2C, right panel). The same was true when all the vaccinated animals were compared with the control group (p = 0.003) (Fig. 2B, right panel).

Evaluation of SIV-mediated gastrointestinal α4β7high+/CD4+ T cell population depletion postinfection

Profound depletion of intestinal CD4+ T cells in SIV-infected macaques occurs rapidly postinfection. Because there is a direct correlation between the levels of systemic α4β7high+/CD4+ Cm T cells and the number of the same cell population in the gastrointestinal mucosa, the evaluation of α4β7high+/CD4+ Cm T cells in PBMCs is an indication of how the virus is affecting this population in the gastrointestinal tract (43).

The effect of pre-existing immunity to SIV on virus-mediated depletion of α4β7high+/CD4+ Cm T cells was evaluated after early infection by measuring the number of α4β7high+/CD4+ Cm T cells in PBMCs in all the vaccinated and control animals (Fig. 3). During the acute phase of the infection (week 2–20), when the gastrointestinal depletion is at its peak, the percentage average of circulating α4β7high+/CD4+ Cm T cells was significantly lower in the control group compared with the vaccinated animals (p = 0.004, Mann–Whitney U test, two-tailed p value) (Fig. 3C). When each of the vaccinated group was compared with the control group, a higher percentage of α4β7high+/CD4+ Cm T cells was detected in group 1 and group 2 (p = 0.0005 and p = 0.00006 respectively) (Fig. 3D). We investigated whether early preservation of α4β7high+/CD4+ Cm T cells, which may parallel a better later preservation of the immune system in general, can be asso-

FIGURE 5. Levels of immune activation in CD4+ and CD8+ T cell memory populations postinfection. A, CD38high+/CD4+ Cm (top) and EM (bottom) T cell percentage averages in the control group (green) and in the vaccinated animals (black) (left panels), or in each of the three groups (right panels); time of death refers to the time of euthanasia, which was due to AIDS in 10 animals and occurred at different time points, or was at week 130 for the healthy animals, euthanized because of closure of the experiment. The first number in the parentheses at the end of each graph line indicates the number of animals in that group that developed AIDS, and the second number indicates the number of animals in that group. B, CD38high+/CD8+ Cm and EM T cell percentage averages in the control group and in all vaccinated animals (left panels), in each of the three groups (right panels). C, Correlation between plasma viral load at 20 wk (left) or at 60 wk postinfection (right) and percentages of CD38high+/CD4+ Cm T cells. D, Correlation between viremia at week 20 (left) or at week 60 postinfection (right) and percentages of CD38high+/CD8+ Cm T cells. E, Correlation between CD38high+/CD8+ Cm T cell percentage at 20 wk postinfection and CD4+ Cm T cell percentage at week 16 postinfecition (left) or between CD38high+/CD8+ Cm T cell percentage at week 20 postinfection and survival (right). F, Kaplan–Meier survival curves shown for all animals after dividing them into two groups according to the CD38high+/CD8+ Cm T cell percentage at 20 wk postinfection (the 9 higher and 10 lower levels of CD38high+/CD8+ Cm T cells).
associated with long-term control of viremia by evaluating the number of $\alpha_4\beta_7^{high+/}CD4^+$ CM T cells early in infection in the animals with the best and worst viral loads at week 60 postinfection. As expected, a significant preservation of $\alpha_4\beta_7^{high+/}CD4^+$ CM T cells was observed in the animals that maintained low or undetectable viremia long term ($p = 0.01$) (Fig. 3E). Moreover, a significant inverse correlation was observed between the percentage of $\alpha_4\beta_7^{high}$ CD4$^+$ CM T cells 12 wk postinfection and viremia levels at week 60 postinfection (Fig. 3F). These results suggest that early preservation of gastrointestinal CD4$^+$ CM T cells can predict long-term control of viral replication.

Preservation of circulating CD4$^+$ T cell memory
Preservation of the CD4$^+$ CM T cell population has been shown to correlate with better control of viral replication and disease progression (7, 44). Similarly, the postinfection time course analysis of the circulating CD4$^+$ memory T cell population revealed significantly higher percentages of total, CM, and EM CD4$^+$ T cells when the average of the values measured at week 16 postinfection in all vaccinated animals was compared with that of the control group (total memory, $p = 0.02$; CM, $p = 0.01$; EM, $p = 0.03$; Mann–Whitney U test, two-tailed p value) (Fig. 4A, top panels). When the comparison was made between average values of individual groups, both immunized groups had higher levels of total memory CD4$^+$ T cells (group 1 versus control, $p = 0.05$; group 2 versus control, $p = 0.04$) (Fig. 4A, bottom left panel) and of CD4$^+$ CM T cells than did the control (group 1 versus control, $p = 0.02$; group 2 versus control, $p = 0.04$) (Fig. 4A, bottom middle panel). Differences in the levels of CD4$^+$ EM T cells were not statistically significant between groups (Fig. 4A, bottom left panel).

Severe loss of the gastrointestinal CD4$^+$ memory T cell population is observed early after HIV and SIV infection (45, 46). This population was evaluated in MNCs isolated from rectal biopsies 8 wk postinfection. No significant differences in the CD4$^+$ total memory T cells were detected between groups (Fig. 4B, left panel). However, a significant loss of the rectal mucosa CD4$^+$ CM T cells was detected in group 2 and the control group, compared with group 1 (group 1 versus control, $p = 0.02$; group 1 versus group 2, $p = 0.02$; Fig. 4B, middle panel; Mann–Whitney U test, two-tailed p value). Taken together, these data suggested that a better preservation of the gastrointestinal CD4$^+$ CM T cell population was elicited by the IL-2/IL-15 adjuvant combination than by IL-12/GM-CSF/TNF-α. As depletion of the CD4$^+$ CM T cell population is associated with disruption of the mucosal barrier, microbial translocation, and immune activation, this preservation may impact progression to AIDS.

After dividing the animals into two groups, according to CD4$^+$ CM T cell levels at week 16 postinfection (CD4$^+$ CM $> \text{or} < 26.5\%$)
or according to the viremia control at week 20 postinfection (10 animals with \( \leq 1.1 \times 10^5 \) copies/ml and 9 animals with \( \geq 2.2 \times 10^5 \) copies/ml), only 2 of the 10 animals in the higher CD4⁺ CM T cell percentage group developed AIDS during the time course of the trial (median survival >130; Fig. 4C, left panel). In contrast, all 9 animals in the group with lower CD4⁺ CM T cell percentage developed AIDS, and the median survival was 70 wk postinfection (\( p = 0.01 \), Mantel–Cox log-rank test). Similarly, only 1 animal of the 10 in the group with better viremia control developed AIDS, whereas all the animals in the group with poor viremia control developed AIDS and the median survival was 63 wk postinfection (\( p = 0.0001 \); Fig. 4C, right panel).

In summary, these data confirm the results we and others have observed in the rhesus male model and in elite controllers: The early preservation of the CD4⁺ CM T cell population and the better control of viremia are associated with long-term survival. Given that all six nonvaccinated animals ranked in the groups with poor control of disease progression, we can conclude that our vaccines provided protection from disease progression regardless of the adjuvant administered.

**Impact of immunization on systemic immune activation postinfection**

Immune activation occurs more significantly during lentiviral infection in species that progress to AIDS compared with primate species that do not develop AIDS. We reasoned that if a vaccine provides protection from disease progression it should also reduce immune activation during the asymptomatic phase of the infection. The magnitude of immune activation was investigated by evaluating the fraction of CM and EM CD4⁺ and CD8⁺ T cells that expressed high levels of the CD38 marker, a membrane-bound protein with an ADP-ribosyl cyclase function, whose increased expression has been linked with T cell activation (47, 48). The analysis was carried out by flow cytometry on PBMCs from week 2 postinfection to time of death (Fig. 5). A more significant increase of CD4⁺/CD38⁺ CM and EM T cell percentage was observed in PBMCs from control animals compared with PBMCs from immunized animals (\( p = 0.0011 \), Mann–Whitney U test, two-tailed \( p \) value; Fig. 5A, left panel). Significantly lower levels of immune activation were observed in circulating CD4⁺ CM and EM T cells of group 1 and group 2 animals compared with controls (CM: \( p = 0.03 \), \( p = 0.04 \); EM: \( p = 0.003 \), \( p = 0.05 \); Fig. 5A, right panel). The vaccine-mediated reduction of immune activation was even more striking when evaluated in the CD8⁺ T cell populations (Fig. 5B). Significantly lower levels of immune activation were detected when CD8⁺/CD38⁺ CM and EM T cell average numbers in all vaccinated animals were compared with the numbers in control animals (\( p = 0.03 \); Fig. 5B, left panel) and each of the vaccinated groups was compared with the controls (group 1 versus control, \( p = 0.03 \); group 2 versus control, \( p = 0.046 \); Fig. 5B, right panel).

Furthermore, a positive correlation was detected between immune activation, measured at week 20 postinfection as percentage of CD4⁺/CD38⁺ CM or CD8⁺/CD38⁺ CM T cells, and viral loads, whether measured early in the chronic infection (week 20) (\( p = 0.039 \) and \( p = 0.01 \), respectively; Spearman’s rank correlation coefficient) or at a later time point (week 60) (Fig. 5C, \( p = 0.0008 \).

**FIGURE 7.** Correlates of protection. A, Correlation between systemic SIV-specific CD4⁺/IFN-\( \gamma \)⁺ and CD8⁺/IFN-\( \gamma \)⁺ T cell responses detected on day of challenge and long-term control of viremia (week 60 postinfection) or survival. SIV-specific cell-mediated responses in PBMC postinfection. B, Average and SEM of CD4⁺ or CD8⁺ Gag + Env-specific T cell percentages obtained for each animal group. C, Average and SEM of CD4⁺ or CD8⁺ Gag plus Env-specific T cell percentages obtained for all vaccinated animals and control animals. D, Average and SEM of CD4⁺ or CD8⁺ Gag plus env-specific T cell percentages obtained after dividing the animals into two groups according to the CD4⁺ CM T cell percentage at week 16 (the 10 highest and the 9 lowest levels).
and $p = 0.007$, respectively). In contrast, a negative correlation was detected between CD8$^+$ CM immune activation at week 20 and survival or CD4$^+$ CM T cell numbers 16 wk postinfection (Fig. 5E, $p = 0.005$ and $p = 0.05$, respectively). When Kaplan–Meier curves for the animals divided into two groups (9 higher and 10 lower CD8$^+$ T cell immune activation at week 20 postinfection) were plotted, significantly longer survival was observed in the group with lower levels of immune activation (Fig. 5F, $p = 0.04$, Mantel–Cox log-rank test). The lower level of immune activation maintained postinfection in the immunized animals is an additional marker of vaccine efficacy and is consistent with the fact that immune activation is driven by viremia and affects disease progression.

Postinfection antiviral immune responses and immunological correlates of protection

As nasal administration of two AIDS vaccine regimens showed efficacy in female rhesus macaques, we investigated whether any of the antiviral responses detected on the day of challenge or postinfection correlated with reductions in viral load and protection against disease progression.

No significant correlation was observed between levels of SIV-specific Abs present on the day of challenge and viremia control or survival. Postinfection plasma virus-specific IgG titers and neutralizing Ab titers reflected the amount of viral load detected in the serum, with viremic animals in all three groups developing similar

**FIGURE 8.** SIV-specific cell-mediated immune responses in rectal and vaginal mucosa MNCs postinfection. Animals were divided into two groups of 10 and 9 animals, based on percentages of CD4$^+$ CM T cells 16 wk postinfection. Percentages of SIV Gag + Env-specific CD4$^+$ and CD8$^+$ T cell responses detected in MNCs isolated from rectal biopsies (A) or vaginal biopsies (B) at week 8 (represented in blue or purple shades) and 32 (represented in brown or teal shades) postinfection are shown for each animal. Left panels show the percentage of cells producing one cytokine of the three tested (IL-2 or IFN-$\gamma$ or TNF-$\alpha$) upon stimulation with SIV peptide pools (monofunctional responses), and right panels illustrate the percentage of cells with simultaneous production of IFN-$\gamma$/TNF-$\alpha$ or IL-2/IFN-$\gamma$ or TNF-$\alpha$/IL-2 or IFN-$\gamma$/TNF-$\alpha$/IL-2 (multifunctional responses). The order of animal numbers in the x-axis is according to rank, from the animal with the highest CD4$^+$ CM T cell value to the one with the lowest. The color of the number indicates the group to which the animal belongs according to the legend of Fig. 2. The number of the animals that controlled viremia to undetectable levels is underlined.
total anti-SIV IgG and neutralizing titers 20–32 wk postchallenge (Fig. 6A, 6B). Humoral responses to the viral envelope could not be associated with viral control because protected animals had no anti-env Abs in serum or secretions just before challenge, and postinfection, most did not mount Ab responses at all (Fig. 6). In contrast, vaccinated animals that failed to control viremia developed large increases of systemic and mucosal anti-SIV Abs postinfection, which were very similar in kinetics and magnitude to those in naive controls (Fig. 6). Interestingly, all of the animals that had Gag-, Pol-specific IgA in secretions or Gag-, Pol-specific IgG in plasma just before challenge were found to be protected (Fig. 6B, 6C, 6E). Other protected animals had no Abs at all. Thus, no association with protection could be made. However, as multiple mechanisms may contribute to protection and Abs can mediate control of virus replication not only via neutralization but also, for instance, via Ab-dependent cell-mediated cytotoxicity (49, 50), we cannot rule out that these IgA responses played a role in the protection of some animals.

The percentages of SIV-specific CD4+IFN-γ+ and CD8+IFN-γ+ T cell responses (these numbers indicate all IFN-γ–positive cells, whether monofunctional or multifunctional) detected on the day of challenge inversely correlated with the viral loads detected 60 wk postinfection and directly correlated with survival (Fig. 7A), supporting a significant role for these responses in the control of viral replication.

When anti-SIV systemic cell-mediated immunity was analyzed, no significant differences were observed among the three groups (Fig. 7B). To compensate for the small size of the groups that may prevent detection of significance in the antiviral cellular-mediated response, the vaccinated animals were grouped together and compared with the controls. A significantly higher percentage of anti-SIV Gag + Env CD4+IL-2+, CD4+IFN-γ+, CD8+TNF-α+ T cells was detected in vaccinated macaques (p = 0.02, p = 0.01, and p = 0.04, respectively, Mann–Whitney U test, two-tailed p value; Fig. 7C). When the animals were divided into two groups of 10 and 9 animals, based on the percentage level of the CD4+CM T cell 16 wk postinfection, a significantly higher percentage of anti-SIV Gag + Env total monofunctional (IL-2, IFN-γ, and TNF-α) CD4+ and CD8+ T cells and total multifunctional CD8+ T cells was detected in the 10 macaques with better preservation of the CD4+CM T cell population (p = 0.0034, p = 0.01, and p = 0.03, respectively; Fig. 7D), supporting the role of these responses in protection.

When antiviral responses measured on rectal (Fig. 8A) and vaginal mucosa (Fig. 8B) at week 8 and 32 postinfection were compared between the 10 animals with higher and the 9 animals with lower CD4+CM T cell levels, the only significant difference was observed for the vaginal CD8+ T cell monofunctional responses detected at 32 wk postinfection (Fig. 8A; p = 0.02, Mann–Whitney U test, two-tailed p value), which were higher in the group with better preservation of CD4+CM T cells. As only two time points are available for these parameters, it is possible that a more systematic analysis would have revealed a different set of immune correlates of protection than that which we detected. As observed previously, these data indicate that cell-mediated responses appear to be more critical than humoral responses in protecting from CD4+ T cell memory loss and progression to disease.

In the seven animals that controlled viremia to undetectable levels, anti-SIV cell-mediated immune responses were detectable at many time points postinfection (Fig. 9A). SIV-specific PBMC cell-mediated responses were of low magnitude on the day of challenge, increased after viremia was detected, and persisted at different time points during the follow-up, providing additional evidence for the occurrence of the infection with consequent boosting of the immune responses induced by vaccination. Rectal and vaginal cell-mediated responses were also present in these animals and in some cases were at higher levels than when analyzed before challenge or on the first time point postchallenge, supporting the role of additional antigenic stimulation (Fig. 8, underlined numbers). However, plasma anti-SIV Ab titers were negative, with the exception of one animal that had very low anti-SIV IgG titers, and cervicovaginal IgA levels were detectable at multiple time points in four of these animals and became positive at later time points in three of them, arguing in favor of a more significant viral replication having occurred at the site of exposure than systemically.

Discussion

In this study, we have shown that SIV disease progression in female rhesus macaques can be significantly delayed by vaccination that includes an SIV and cytokine DNA plasmids and rMVA expressing SIV Gag, Pol, and Env. Lower or undetectable viremia, better preservation of α4β7 and CM CD4+ T cells, and lower levels of immunoactivation were all observed in the vaccinated groups, compared with controls.

The DNA prime/MVA boost vaccine approaches chosen in these experiments stimulated significant and consistent anti-SIV cell-mediated responses mucosally, both vaginally and rectally, and systemically, supporting the validity of this platform to achieve anti-SIV cell-mediated responses in both immune compartments and at two mucosal sites where virus transmission in humans occurs. They also confirm the validity of nasal administration of this vaccine to stimulate these particular responses.

Previous studies conducted in male macaques using the simian HIV or SIV-DNA/rMVA vaccine approach administered via mucosal routes, with the addition of IL-2 and IL-15 DNA in some groups, showed efficacy in stimulating cell-mediated responses and delaying disease progression but failed at stimulating consis-

**FIGURE 9.** A. Postinfection CD4+ and CD8+ Gag plus Env-specific T cell percentages detected in the seven animals that fully controlled viremia long term. B. Humoral responses postinfection in the same animals.
tient humoral responses (3, 4, 7). Furthermore, immunity in the genital tract was not evaluated. In this study in female macaques, both regimens failed to stimulate consistent and sustained humoral responses, despite the fact that one vaccine approach included as adjuvants the cytokines IL-12 and GM-CSF, which we had hypothesized may better stimulate B cell activity and in particular IgA production (27, 29, 31, 34, 35). Modifications of these regimens with the goal of stimulating humoral anti-SIV immunity in addition to cell-mediated immunity need to be explored to achieve a regimen that is optimal at stimulating both.

The addition of IL-2 and IL-15 DNA to the SIV DNA was previously proven to increase the immunogenicity of the SIV DNA given alone (29, 31–33). The comparison of a regimen including these two cytokines with one including GM-CSF, IL-12, and TNF-α did not reveal major differences in the stimulated Ag-specific cell-mediated immunity. A statistically significant difference in levels of rectal mucosa CD4⁺ CM T cells evaluated postinfection was observed between groups 1 and 2, but the overall protection provided postinfection was similar. It is possible that the size of the groups prevented detection of statistically significant differences that do exist between the two groups or, alternatively, that the two cytokine regimens lead to a similar modulation of the cell-mediated response, with consequent similar outcome for the vaccination.

With the exception of one animal that resisted 35 vaginal challenges, prevention of systemic infection was not observed in this trial. The simplest explanation is that sustained humoral responses at the site of virus exposure are necessary for this protection, and these responses were not present in the animals at the time of challenge. It is also possible that, as the amount of virus used for the low-dose challenge is still higher than what is present in human body fluids during natural exposure, the immunity induced by vaccination could contain a smaller virus amount.

The effectiveness of the vaccination impacted multiple parameters that have been associated with virus pathogenicity. Not only was the viremia reduced or fully controlled and early preservation of CD4⁺ CD4⁺ T cells and reduced immune activation. As CD4⁺ T cell depletion of the gastrointestinal mucosa and immune activation both contribute to disease progression (45, 46), it is not a surprise that vaccination extended the time to AIDS in the vaccinated animals. Furthermore, these findings indicate that measurements of blood levels of CD4⁺ CD4⁺ CM T cells and of CD8⁺ T cell immune activation can provide additional markers besides viremia and early preservation of CM to establish the effectiveness of vaccination early after an infection has occurred. The use of multiple markers could be important when an AIDS vaccine is evaluated in the genetically heterogeneous human population, as it should prevent the confounding effects introduced by outlier data that can occur with the analysis of only one parameter.

Half of the vaccinated and infected animals had a short-lived viremia, subsequently controlled to undetectable levels, and maintained this control long term. This outcome is particularly important, as a highly effective control of viremia in elite controllers has been associated with lack of disease progression. Achieving this result in humans could lead to significantly longer survival of infected individuals and very likely to decreased risk of transmission. It is likely that containment of the infection during the acute stage was due to the immunological control provided by these responses.

In the animals that controlled viremia to undetectable levels, anti-SIV cell-mediated immune responses were detectable at many time points postinfection, and anti-SIV IgA at some time points, whereas IgG titers were negative (Fig. 9). Although persistent infection could be associated with observed cell-mediated responses even if the viremia returns to undetectable levels, lack of development of significant anti SIV IgG titers suggests that the absence of a sustained chronic infection, and possibly viral clearance, are more likely in these animals. This picture of macaques having positive antiviral systemic cell-mediated immunity and positive vaginal IgA, with negative plasma Ab titers and viremia, is reminiscent of immunological findings in individuals at high risk for HIV infection and defined as exposed but uninfected. It is possible that these individuals may actually have been infected at some point but manage to control the viremia to undetectable levels (51–53).

Although the results of the Merck clinical trial were discouraging (11, 12), those obtained with the more recent Thai trial RV144 brought renewed cautious optimism to the development of an AIDS vaccine (13). If some of the immune responses stimulated by the vaccine used in RV144, yet to be fully identified, can be effective at preventing infection in a fraction of individuals, and cell-mediated responses, such as those reported in this article, can control viremia to undetectable levels in individuals that become infected, one could reasonably hope that, with additional improvements or combinations of these approaches, the effort to achieve control of the AIDS epidemic is no longer a Sisyphean task.

Disclosures
The authors have no financial conflicts of interest.

References