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Venkateswarlu Chamcha, Emory University
Sunil Kannanganat, Emory University
Sailaja Gangadhara, Emory University
Rafiq Nabi, Louisiana State University
Pamela A. Kozlowski, Louisiana State University
David C. Montefiori, Duke University
Celia C. LaBranche, Duke University
Jens Wrammert, Emory University
Brandon F. Keele, Frederick National Laboratory for Cancer Research
Harikrishnan Balachandran, Beth Israel Deaconess Medical Center

Only first 10 authors above; see publication for full author list.

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Strong, but Age-Dependent, Protection Elicited by a Deoxyribonucleic Acid/Modified Vaccinia Ankara Simian Immunodeficiency Virus Vaccine

Venkateswarlu Chamcha,1 Sunil Kannanganat,1 Sailaja Gangadhara,1 Rafiq Nabi,2 Pamela A. Kozlowski,2 David C. Montefiori,2 Celia C. LaBranche,3 Jens Wrammert,4 Brandon F. Keele,5 Harikrishnan Balachandran,6 Sujata Sahu,6 Michelle Lifton,6 Sampa Santra,6 Rahul Basu,7 Bernard Moss,8 Harriet L. Robinson,7 and Rama Rao Amara1

1Yerkes National Primate Research Center, Emory University, Atlanta, Georgia; 2Department of Microbiology, Immunology and Parasitology, Louisiana State University Health Sciences Center, New Orleans; 3Duke University Medical Center, Durham, North Carolina; 4Department of Pediatrics, Emory University School of Medicine, Atlanta, Georgia; 5AIDS and Cancer Virus Program, Leidos Biomedical Research, Inc., Frederick National Laboratory for Cancer Research, Maryland; 6Harvard Medical School, Beth Israel Deaconess Medical Center, Boston, Massachusetts; 7Geovax Inc, Smyrna, Georgia; 8Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland

Background. In this study, we analyzed the protective efficacy of a simian immunodeficiency virus (SIV) macaque 239 (SIVmac239) analogue of the clinically tested GOVX-B11 deoxyribonucleic acid (DNA)/modified vaccinia Ankara (MVA) human immunodeficiency virus vaccine.

Methods. The tested vaccine used a DNA immunogen mutated to mimic the human vaccine and a regimen with DNA deliveries at weeks 0 and 8 and MVA deliveries at weeks 16 and 32. Twelve weekly rectal challenges with 0.3 animal infectious doses of SIV sootey mangabey E660 (SIVsmE660) were administered starting at 6 months after the last immunization.

Results. Over the first 6 rectal exposures to SIVsmE660, <10-year-old tripartite motif-containing protein 5 (TRIM5α)-permissive rhesus macaques showed an 80% reduction in per-exposure risk of infection as opposed to a 46% reduction in animals over 10 years old; and, over the 12 challenges, they showed a 72% as opposed to a 10% reduction. Analyses of elicited immune responses suggested that higher antibody responses in the younger animals had played a role in protection.

Conclusions. The simian analogue of the GOVX-B11 HIV provided strong protection against repeated rectal challenges in young adult macaques.

Keywords. age-dependent protection; antibody; DNA/MVA vaccine; HIV vaccine; SIV.
immunogen more like the DNA in GOVX-B11 and the regimen closer to the updated regimen being advanced with GOVX-B11. In particular, the DNA prime was modified by an inactivating point mutation in protease to enhance VLP production by preventing premature cleavage of overexpressed Gag [9]. The regimen was modified to favor avidity maturation of the Ab response by allowing 16 instead of 8 weeks between the 2 MVA boosts [10]. The regimen for the clinical advancement of GOVX-B11 is 2 DNA primes at 0 and 8 weeks followed by 3 MVA boosts at 16, 24, and 40 weeks. The trial also differed from prior trials by including 3- to 16-year-old male and female macaques as opposed to only 3- to 5-year-old males. Rhesus macaques reach puberty at 3 to 4 years of age with 1 year of rhesus life being approximately equivalent to 4 years of human life (http://genomics.senescence.info/species/entry.php?species=Macaca_mulatta). This means that trials that had been previously conducted in adolescents were now being conducted in young, middle-aged, and even elderly macaques.

The results of this study revealed the induction of higher avidity Abs than in the prior trial and similar avidity and protection in the GM-CSF-adjuvanted and nonadjuvanted groups. In TRIM5α-permissive animals <10 years old, an 80% reduction in per-exposure risk of infection occurred over the first 6 exposures, and a 72% reduction occurred over all 12 exposures. On the basis of these findings, the nonadjuvanted GOVX-B11 vaccine will undergo further development using a 16-week rest between the final 2 MVA boosts, and efficacy testing will limit participants to youths and young adults.

METHODS

Vaccines

The SIVmac239 DNA vaccines used for priming the immune response were modified from the Rama36 (non-GM-CSF coexpressing) and Rama42 (GM-CSF coexpressing vaccines) used in the prior study [5] by mutating the active site of protease from an aspartic acid to alanine (D26A) [9]. The resulting non-GM-CSF coexpressing DNA (Rama33) and GM-CSF coexpressing DNA (Rama47) expressed SIVmac239 Gag, PR, RT, Env, Tat, and Rev from a single ribonucleic acid (RNA) by subgenomic splicing and frame shifting. GM-CSF was expressed by the same mRNA as Env using the encephalomyocarditis virus internal ribosome entry site [5]. Levels of GM-CSF expression were measured on 48-hour supernatants of transiently transfected HEK293T cells using an enzyme-linked immunosorbent assay (ELISA) for human GM-CSF (Mabtech, Inc., Cincinnati, OH). The protease-inactivated Rama47 expressed slightly higher levels of GM-CSF than the protease-active Rama42 used in the previous study (617 ± 295 units compared with 407 ± 107 units). The same recombinant MVA as used before (SIVmac239-MVA formally designated DR1 or MVASIVgpe) expressed Gag, Pol, and Env but did not coexpress GM-CSF [11]. The DNA vaccines expressed the complete gp160 form of SIVmac239 Env, and the MVA vaccine encoded a gp150 form, which was truncated to remove 146 amino acids at the C-terminus of the transmembrane subunit to enhance expression and stabilize the insert [2].

Animals and Challenge Stock

Sixty Indian origin rhesus macaques (Macaca mulatta) weighing from 2.7 to 15.5 pounds were housed at Bioqual Inc. and cared for under guidelines established by the Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals using protocols approved by the Institutional Animal Care and Use Committee. The challenge stock, SIVsmE660-ABL, was grown from the stock used in the prior trial (designated SIVsmE660-Hirsch 2000) [5] using peripheral blood mononuclear cells from the same pigtailed macaque used to produce the original challenge stock. All immunological and tests for infection were performed blinded. Sequence analysis and enumeration of transmitted-founder genomes was determined as previously described [12].

Antibody Responses

Simian immunodeficiency virus-specific Ab responses were assessed in serum by Env-specific ELISAs using commercially purchased SIVmac239 gp140 antigen (Immune Technology Corp, New York, NY) as previously described [13, 14]. The concentrations of immunoglobulin (Ig)G were estimated relative to a standard curve. Rectal secretions were collected using premoistened Weck-Cel Sponges as previously described [15]. For mucosal Abs, Env-specific IgG or IgA was measured and represented as nanograms of specific Ab per microliters of total Ab [14]. TZM-bl luciferase-based neutralization assays were performed with tier 1 and tier 2 viruses as described previously [16]. Antibody-secreting cells were measured by enzyme-linked immunospot assays as previously described [17] using SIVmac239 gp140 as a coating antigen. Avidity of binding Ab was determined for SIVmac239 gp160 captured from VLP produced by transient transfection of HEK293T cells with Rama33 as described previously [14]. A reference standard of pooled sera was used in all assays. This standard had a mean avidity index of 52 and a standard deviation of 1.2. Antibody-dependent cellular phagocytosis (ADCP) assays were performed using THP-1 monocytic cells and SIVsmE660 gp140-coated fluorescent beads, and the phagocytic score was calculated as described previously [18]. Antibody-dependent cellular cytotoxicity (ADCC) assays were performed as described earlier [19] using SIVmac239-infected CEM.NKR-CCR5 CD4+ T cells as target cells.

T Cell Responses

Simian immunodeficiency virus-specific cellular immune responses were assessed by multiparameter intracellular cytokine staining (ICS) assays after stimulation with SIVmac239 peptides as previously described [20]. All values used in the analyses
were background subtracted and 2 times higher than background levels.

### Statistical Analysis
Kaplan-Meier curves and the log-rank Mantel Cox test were used to display and test for differences in infection curves. Per-exposure reductions in risk of infection were analyzed as described by Hudgens and Gilbert [21, 22]. The Wilcoxon Mann-Whitney U test was used to compare Ab and T-cell responses and viral RNA levels between groups. The Spearman rank correlation method was used for correlations. P values were not corrected for multiple comparisons, and a 2-sided P value of .05 was considered significant. Statistical analyses were performed using GraphPad Prism version 6.0 for Mac (GraphPad Software, San Diego, CA) and TIBCO Spotfire S 8.1 (TIBCO, Somerville, MA).

### RESULTS

#### Study Design
Thirty male and 30 female animals were randomized by sex and weight into 2 vaccine and 1 control group of 20 each. Rhesus in the vaccine groups were immunized intramuscularly in the quadriceps at 0 and 8 weeks with 3 mg of SIVmac239-DNA in phosphate-buffered saline (PBS) either coexpressing (Dg) or not coexpressing GM-CSF (D) followed by boosting with $1 \times 10^8$ plaque-forming units (pfu) of SIVmac239-MVA (M) in PBS at 16 and 32 weeks (DDMM or DgDgMM regimens) (Figure 1A). The control group received $1 \times 10^8$ pfu of parental MVA at 16 and 32 weeks. At 6 months after the final immunization, animals were entered into 12 weekly rectal exposures to 412 tissue culture infection dose (TCID$_{50}$) of SIVsmE660-ABL. At each challenge, animals were tested for infection and considered infected when an animal scored for >1000 copies of viral

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**Figure 1.** Schematic of trial: Kaplan–Meier curves for vaccine-elicited prevention of infection and vaccine efficacy. (A) Schematic showing the vaccination and challenge schedule for the trial. (B) Kaplan-Meier curves for prevention of infection in all vaccinated and control animals separated by age. Granulocyte-macrophage colony-stimulating factor (GM-CSF) adjuvanted and nonadjuvanted vaccine groups were pooled for analysis. (C) Kaplan-Meier curves for prevention of infection in tripartite motif-containing protein 5 (TRIM5α)-permissive animals separated by age. (D) Vaccine efficacy. (E) Number of transmitted variants. For details, see Methods. Abbreviations: CI, confidence interval; Cont, controls; All Vac, combined groups; shaded area, first 6 challenges.
RNA/mL plasma on 2 consecutive bleeds or >5000 copies of viral RNA/mL on a single bleed. Once an animal was infected, further challenges were stopped. Animals were typed for TRIM5α alleles [23, 24]. Animals that were TFP/TFP or TFP/CypA were considered TRIM5α restrictive, whereas animals that were TFP/Q, CypA/CypA, Q/CypA, or Q/Q were considered permissive.

**Strong Vaccine Mediated Protection in Younger but Not Older Tripartite Motif-Containing Protein 5α-Permissive Animals**

Animals vaccinated with both the adjuvanted and nonadjuvanted vaccines were protected against serial rectal challenges with SIVsmE660 (Supplementary Figure 1). In contrast to our prior study, animals primed with the nonadjuvanted vaccine showed the same level of protection as those primed with the GM-CSF co-expressing vaccine (Supplementary Figure 1A). As expected, TRIM5α-restrictive animals showed better protection than TRIM5α-permissive animals (Supplementary Figure 1). Given that indistinguishable levels of protection had occurred in the adjuvanted and nonadjuvanted groups, the 2 groups were pooled for further evaluation.

Because age can significantly influence vaccine-induced immune responses [25–29], animals in the study were next analyzed for effects of age on protection (Figure 1). Two groups were established: the first more than 10 years old (n = 26) and the second <10 years old (n = 14).

The younger animals showed highly significant protection (P = .002), whereas the older animals did not (Figure 1B). In younger animals, per-challenge reductions in risk of infection were 76% over the first 6 challenges and 66% over all 12 challenges (Figure 1D). For younger TRIM5α-permissive animals, there was an 80% reduction in per-challenge risk of infection during the first 6 challenges and a 72% reduction in per-challenge risk over all 12 challenges (Figure 1C and 1D). In contrast, for TRIM5α-permissive animals more than 10 years old, the per-exposure risk reduction was 46% over the first 6 challenges and only 10% over all 12 challenges (Figure 1C and 1D). Analysis of 10 vaccinated and 10 unvaccinated TRIM5α-permissive animals for the number of transmitted-founder variants revealed a median of 1 (range, 1–3) in the vaccinated animals and a median of 2 (range, 1–7) in the unvaccinated animals (Figure 1E).

**Immune Correlates for Protection**

Elicited Ab responses for SIVmac239 gp140 had similar temporal magnitudes in the GM-CSF-adjuvanted and nonadjuvanted groups (Figure 2). Combining data for both vaccinated groups, the estimated median level of Env-specific IgG in sera at 2 weeks
Table 1. Summary of Elicited Responses and Correlations With Number of Challenges to Infection

<table>
<thead>
<tr>
<th>Immunological Assay Performed</th>
<th>Peak Value</th>
<th>Prechallenge Value</th>
<th>ρ Value</th>
<th>P Value</th>
<th>Peak Value</th>
<th>Prechallenge Value</th>
<th>r Value</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIVmac239 gp140-specific IgG in serum (µg/mL)</td>
<td>241.5 (181, 327)</td>
<td>37 (26, 37)</td>
<td>0.32</td>
<td>.04</td>
<td>241.5 (181, 327)</td>
<td>37 (26, 37)</td>
<td>0.32</td>
<td>.03</td>
</tr>
<tr>
<td>SIVmac239 gp140-specific IgG in rectal swabs (ng of specific IgG/µg IgG)</td>
<td>11.2 (7.5, 18.23)</td>
<td>1.6 (0.7, 2.39)</td>
<td>0.37</td>
<td>.10</td>
<td>11.2 (7.5, 18.23)</td>
<td>1.6 (0.7, 2.39)</td>
<td>0.37</td>
<td>.10</td>
</tr>
<tr>
<td>SIVmac239 gp140-specific IgG secreting ASCs (ASCs/10^6 PBMCs)</td>
<td>116 (52.5, 223.5)</td>
<td>60 (18, 90)</td>
<td>0.53</td>
<td>.01</td>
<td>116 (52.5, 223.5)</td>
<td>60 (18, 90)</td>
<td>0.53</td>
<td>.01</td>
</tr>
<tr>
<td>SIVmac239 gp140-specific IgG secreting ASCs (ASCs/10^6 PBMCs)</td>
<td>45 (22.5, 82.0)</td>
<td>18 (9, 24)</td>
<td>0.63</td>
<td>.02</td>
<td>45 (22.5, 82.0)</td>
<td>18 (9, 24)</td>
<td>0.63</td>
<td>.02</td>
</tr>
<tr>
<td>SIVmac239 gp140-specific IgA secreting ASCs (ASCs/10^6 PBMCs)</td>
<td>0.63 (0.38, 1.15)</td>
<td>0.34</td>
<td>.03</td>
<td></td>
<td>0.63 (0.38, 1.15)</td>
<td>0.34</td>
<td>.03</td>
<td></td>
</tr>
<tr>
<td>Neutralizing titers against challenge virus (SIVsmE660-ABL)</td>
<td>3078 (1440, 4710)</td>
<td>446 (188, 73.3)</td>
<td>0.32</td>
<td>.04</td>
<td>3078 (1440, 4710)</td>
<td>446 (188, 73.3)</td>
<td>0.32</td>
<td>.04</td>
</tr>
<tr>
<td>ADCC activity (AUC values)</td>
<td>2.06 (1.32, 2.57)</td>
<td>0.24</td>
<td>.13</td>
<td></td>
<td>2.06 (1.32, 2.57)</td>
<td>0.24</td>
<td>.13</td>
<td></td>
</tr>
<tr>
<td>SIV-specific IFNγ+CD4+ T-cell response</td>
<td>0.21 (0.11, 0.67)</td>
<td>0.08 (0.05, 0.17)</td>
<td>0.7</td>
<td>.3</td>
<td>0.21 (0.11, 0.67)</td>
<td>0.08 (0.05, 0.17)</td>
<td>0.7</td>
<td>.3</td>
</tr>
<tr>
<td>SIV-specific IFNγ+CD8+ T-cell response</td>
<td>0.35 (0.2, 1.2)</td>
<td>0.04 (0.02, 0.15)</td>
<td>0.1</td>
<td>.5</td>
<td>0.35 (0.2, 1.2)</td>
<td>0.04 (0.02, 0.15)</td>
<td>0.1</td>
<td>.5</td>
</tr>
</tbody>
</table>

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; ASCs, antibody-secreting cells; AUC, area under the curve; gp, glycoprotein; IFN, interferon; Ig, immunoglobulin; PBMCs, peripheral blood mononuclear cells; SIV, simian immunodeficiency virus; SIVmac239, SIV macaque 239.

a Median (25th, 75th percentiles).
b Spearman correlation coefficient.
c Two-tailed P value, unadjusted for Bonferroni correction.

After the second and final MVA boost (week 34) was 241 µg/mL (Table 1). This level had contracted 6.5 fold to a median of 37 µg/mL at week 52, 5 weeks before the initiation of the serial rectal challenges (Table 1). Prechallenge levels of gp140-specific IgA in serum (0.6 µg/mL) were 60-times lower than prechal-
tantal challenges (Table 1). Prechallenge levels of gp140-speci-
cific IgG and IgA ASCs at day 5 after the second MVA boost (Figure 2D) (Table 1). Specific activities for gp140-specific IgA in rectal se-
cretions were measured, but are not presented because these values were at the background for detection.

Correlations conducted on the combined groups for elicited levels of binding Ab, or ASC, and the number of challenges to infection revealed modest but significant direct correlations be-
tween the levels of gp140-specific IgG and IgA in serum, gp140-
specific IgG in rectal secretions and IgG and IgA ASCs, and the number of challenges to infection (Figure 2 and Table 1). The correlation coefficients were lowest for elicited IgG and IgA in prechallenge Sera (r = 0.32, P = .03 and r = 0.34, P = .03, respectively), slightly higher for gp140-specific IgG in rectal secretions prechallenge (r = 0.42, P = .006), and the highest for gp140-
specific IgG and IgA ASCs at day 5 after the second MVA boost (r = 0.53, P = .01 and r = 0.63, P = .02, respectively).

Measurements of the avidity of the Ab response for the SIV-
mac239 gp160 revealed similar high avidities in the 2 groups (Figure 3A). The median avidity index for the pooled data was 49 with a range of 43 to 54. These values were higher than in the prior study in which the GM-CSF group had a me-
dian index of 39 with a range of 29 to 49 and the non-GM-CSF

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with protection (Figure 2), we compared Ab responses in animals that were younger than or older than 10 years of age (Figure 4). These analyses, which included all animals, revealed significantly lower levels of elicited IgG in the older animals (Figure 4). The effect of age on Ab responses was evident in serum (Figure 4A) and rectal secretions (Figure 4B). In addition, older animals had significantly lower neutralizing Ab and ADCC responses (Figure 4C and D). In contrast, age did not significantly affect the levels of elicited T cells (Supplementary Figure 2).

Finally, the effects of vaccination were analyzed for control of viremia during the first 6 months after infection (Figure 5). For these studies, infection was assumed to have taken place 1 week before the first detection of virus. One hundred-fold reductions in peak viremia occurred in the animals that were <10 years old (Figure 5B). These reductions occurred in the group representing all animals under 10 as well as in the TRIM5α-permissive group. No reduction in postchallenge viremia occurred in animals over 10 years of age (Figure 5C). The coexpressed GM-CSF did not appear to have an effect on postinfection control (Figure 5A, left panel). The TRIM5α-restrictive animals showed enhanced viral control compared with TRIM5α-permissive animals both in vaccinated and unvaccinated control groups. Analysis of male and female cohorts did not reveal substantial differences in elicited responses or protection (data not shown).

**DISCUSSION**

In this trial, a SIVmac239 prototype of our GOVX-B11 HIV vaccine showed strong protection against a pathogenic heterosexual SIVsmE660 mucosal challenge. More importantly, this protection was not dependent on the presence of restrictive TRIM5α alleles and did not require GM-CSF as an adjuvant. In addition, our trial showed a profound effect of age on the ability of a SIVmac239 vaccine to raise protective immunity for SIVsmE660. The reductions in per-exposure risk of infection were by far greater in animals <10 years old than in animals more than 10 years old. Over the first 6 rectal exposures, younger TRIM5α-permissive animals had 1.7 times higher reductions in per-challenge risk of infection than older animals (80% as opposed to 46%); and, over the 12 challenges administered in the trial, they had 7 times higher reductions in per-exposure risk of infection (72% as opposed to 10%). The challenge was a rigorous challenge as evidenced by the presence of a median of 2
Figure 4. Effect of age on antibody (Ab) responses. Levels of elicited immunoglobulin (Ig)G are shown on the left and correlations with number of challenges to infection are on the right. (A) Levels of elicited glycoprotein (gp)140-specific IgG in serum. (B) Specific activity of elicited gp140-specific IgG in rectal secretions. (C) Magnitudes of neutralizing Ab against the challenge virus. (D) Antibody-dependent cellular cytotoxicity (ADCC) titer. Responses were measured at peak response (2 weeks after the final modified vaccinia Ankara boost) and prechallenge (week 52). For each panel, the left panels show the magnitudes of responses elicited for all animals in the 2 age groups, and the right panels show the correlation between the elicited Ab at peak and prechallenge timepoints and the number of challenges to infection. In the right panels, the color of points indicates whether data were from a GM-CSF-adjuvanted or nonadjuvanted animal. Abbreviations: AUC, area under the curve; ID₅₀, inhibitory dose for 50% neutralization; Peak, week 34; Pre, preimmunization; pre-chall, week 52; shaded areas, background of detection; SIVmac239, simian immunodeficiency virus macaque 239.
transmitted variants in unvaccinated animals (range of 1 to 7) as opposed to 1 transmitted variant in vaccinated animals (range of 1 to 3). Most heterosexual transmissions have only 1 transmitted variant [30].

Elicited immune responses suggested that Ab, and not T cells, had played the key role in delaying infection. Levels of Env-specific IgG and IgA in serum, the specific activity of Env-specific IgG in rectal secretions, and IgG- and IgA-producing ASC showed modest correlations with the number of challenges to infection. Consistent with the poorer protection in older animals, each of these protection-associated Ab responses was significantly lower in older than younger animals.

Analyses for correlates of protection revealed weak correlations between binding Ab for Env in serum and rectal secretions and the number of challenges to infection. These correlations did not extend to functional assays for neutralizing and non-neutralizing activities. A failure of neutralizing Ab to protect is consistent with our prior studies in the SIV/macaque model [31]. However, others have found correlations between non-neutralizing Fc-mediated mechanisms of protection such as ADCP and ADCC and protection [32–39]. The fact that we did not observe these correlates may reflect the overall similar levels of responses in both vaccine groups, combined with the heterogeneity in age and sex having masked effects that have been observed for vaccines that raised a broader range of responses and were tested in more homogeneous groups [32–38].

The trial was undertaken to further test the hypothesis that avidity of elicited Ab for native Env was a correlate for

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**Figure 5.** Temporal postinfection viremia. (A) Temporal viremia for all animals based on vaccination in the presence or absence of coexpressed granulocyte-macrophage colony-stimulating factor in the deoxyribonucleic acid prime. (B) Temporal viremia for pooled vaccine groups that were <10 years old. (C) Temporal viremia for pooled vaccine groups that were more than 10 years old. In each panel, the left figure presents data for all animals, the middle figure presents data for tripartite motif-containing protein 5 (TRIM5α)-permissive animals, and the right figure presents data for TRIM5α-restrictive animals. Points with significant differences are indicated by asterisks: *P ≤ .05; **P ≤ .01. Abbreviations: All Vac, combined groups; DDMM, DNA at 0 and 8 weeks and MVA at 16 and 32 weeks; DgDgMM, the same regimen for the GM-CSF co-expressing DNA; RNA, ribonucleic acid.
Age-dependent Protection for a SIV DNA/MVA Vaccine

H. L. R. and R. R. A. are inventors on the DNA/MVA technology that has been licensed to GeoVax Inc. by Emory University. H. L. R. is an employee of GeoVax and owns stock in GeoVax. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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