Preexisting Vaccinia Virus Immunity Decreases SIV-Specific Cellular Immunity but Does Not Diminish Humoral Immunity and Efficacy of a DNA/MVA Vaccine


*J Immunol* 2010; 185:7262-7273; Prepublished online 12 November 2010; doi: 10.4049/jimmunol.1000751

http://www.jimmunol.org/content/185/12/7262

Supplementary Material

http://www.jimmunol.org/content/suppl/2010/11/12/jimmunol.1000751.DC1

References

This article cites 61 articles, 34 of which you can access for free at:

http://www.jimmunol.org/content/185/12/7262.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Preexisting Vaccinia Virus Immunity Decreases SIV-Specific Cellular Immunity but Does Not Diminish Humoral Immunity and Efficacy of a DNA/MVA Vaccine

Sunil Kannanganat,†§ Pragati Nigam,*† Prayakumar Velu,*† Patricia L. Earl,† Lilin Lai,* Lakshmi Chennareddi,* Benton Lawson,* Robert L. Wilson,‡ David C. Montefiori,§ Pamela A. Kozlowski,‡ Bernard Moss,† Harriet L. Robinson,*‡ and Rama Rao Amara*

The influence of preexisting immunity to viral vectors is a major issue for the development of viral-vector vaccines. In this study, we investigate the effect of preexisting vaccinia virus immunity on the immunogenicity and efficacy of a DNA/modified vaccinia Ankara (MVA) SIV vaccine in rhesus macaques using a pathogenic intrarectal SIV251 challenge. Preexisting immunity decreased SIV-specific CD8 and CD4 T cell responses but preserved the SIV-specific humoral immunity. In addition, preexisting immunity did not diminish the control of an SIV challenge mediated by the DNA/MVA vaccine. The peak and set point viremia was 150- and 17-fold lower, respectively, in preimmune animals compared with those of control animals. The peak and set point viremia correlated directly with colorectal virus at 2 wk postchallenge suggesting that early control of virus replication at the site of viral challenge was critical for viral control. Factors that correlated with early colorectal viral control included 1) the presence of anti-SIV IgA in rectal secretions, 2) high-avidity binding Ab for the native form of Env, and 3) low magnitude of vaccine-elicited SIV-specific CD4 T cells displaying the CCR5 viral coreceptor. The frequency of SIV-specific CD8 T cells in blood and colorectal tissue at 2 wk postchallenge did not correlate with early colorectal viral control. These results suggest that preexisting vaccinia virus immunity may not limit the potential of recombinant MVA vaccines to elicit humoral immunity and highlight the importance of immunodeficiency virus vaccine achieving early control at the mucosal sites of challenge. The Journal of Immunology, 2010, 185: 7262–7273.

ive vector-based vaccines have become popular for their ability to induce strong cellular and humoral immunity (1–10). However, preexisting immunity to viral vectors has been a major issue for the development of viral-vector vaccines. This has been particularly important for vectors such as adenovirus type 5 (Ad5) because of the high prevalence of Ad5-specific immunity in people around the world (11). Similarly, a significant proportion of the United States population is preimmune to vaccinia virus (VV) because of vaccination for smallpox. Although routine vaccination with VV to prevent smallpox ceased more than 30 y ago, the United States government reinitiated vaccination of certain groups because of perceived bioterrorist threats. Because modified vaccinia Ankara (MVA) is an attenuated strain of VV (12), the anti-VV immunity generated by smallpox vaccine may limit the immunogenicity of MVA-based vaccines.

Preexisting immunity to Ad5, VV, or MVA has been shown to reduce the immunogenicity of the respective recombinant viral vectors in mice (13–16), macaques (17–19), and humans (20, 21). The majority of these studies evaluated the effects on cellular immunity, and very little information is available on humoral immunity. In addition, none of these studies evaluated the consequence of this diminished immunogenicity on the efficacy of HIV vaccines using an appropriate challenge model. Furthermore, the results of a recent human trial for an Ad5-based vaccine revealed a higher rate of HIV infection in uncircumcised males with preexisting Ad5 immunity (22, 23). These results showed preexisting immunity to the vaccine vector affecting the efficacy of an HIV vaccine. Thus, it is important to study the effect of preexisting antivector immunity not only on the immunogenicity but also on the efficacy of a candidate HIV vaccine.

DNA prime and live vector boost vaccines have become popular for their ability to elicit high levels of vaccine-specific cellular and humoral immunity (2, 17, 24–32). Our previous preclinical studies in macaques demonstrated that DNA priming and recombinant modified vaccinia Ankara (rMVA) boosting elicited high frequencies of virus-specific CD4 and CD8 T cells and controlled a pathogenic simian HIV (SHIV) 89.6P challenge (2, 3, 33, 34). The prototype HIV-1 clade B version of this DNA/MVA vaccine...
Materials and Methods

Immunizations and challenge

Young adult Indian rhesus macaques from the Yerkes breeding colony were cared for under guidelines established by the Animal Welfare Act and the National Institutes of Health (Bethesda, MD) Guide for the Care and Use of Laboratory Animals using protocols approved by the Emory University (Atlanta, GA) Institutional Animal Care and Use Committee. Macaques were typed for the Mamu-A*01, Mamu B08, and Mamu B17 alleles as described previously (36–38). Macaques were randomized into three trial groups of eight animals each based on weight and A*01 status. There were four Mamu A*01 macaques in each group. Trial groups were randomized into three inoculation and sampling groups. Of the 24 macaques, 16 were vaccinated with a DNA/MVA SIV vaccine, and 8 were unvaccinated. The DNA and rMAV immunizations were delivered i.m. in PBS using a hypodermic needle in the outer thigh. The DNA immunogen expressed SIV239 Gag-Pol, Env, Tat, and Rev. The DNA immunogen was constructed by replacing the ECoRI–NheI fragment of SHIV DNA construct (39) containing HIV-1 89.6 Tat, Rev, and Env genes with an ECoRI-NheI fragment containing SIV Tat, Rev, and Env. Two MVA recombinants, one expressing SIV239 Gag-Pol (40) and the other expressing SIV239 Env (41), were premixed and used for immunizations. Two DNA inoculations were given on weeks 0 and 8, and two rMAV boosters were given on weeks 16 and 24. The DNA was delivered at 1.2 μg/dose, and the rMAV was delivered at 1 × 10^8 PFU/dose. Of the 16 vaccinated animals, 8 received standard dose of Dryvax (Wyeth Laboratories, Madison, NJ) (10^8 PFU) per percutaneous route 17 mo prior to first DNA inoculation. At 9 mo after the final rMAV booster, animals received an intrarectal challenge with SIVmac251 by using a pediatric feeding tube 15 to 20 cm into the rectum. Dr. Nancy Miller at the National Institutes of Health provided the challenge stock, and 100% naive SIV239 macaques can be infected under these conditions.

SIV Env-specific binding Abs were measured with ELISA using tissue culture-produced SIV Env, captured on a Con A-coated plate as described previously (39). Briefly, PBMCs were prestained with CFSE, and ~1 × 10^6 cells were stimulated in 48-well plates in a volume of 600 μl in RPMI containing 10% human serum at 37°C under 5% CO2 for 6 d. Cells were labeled with p27 Alexa Fluor 700 (clone SK1) and anti-human CD4-PerCP (clone SKD2; Beckman Coulter, Brea, CA) and anti-human CD95-Pacific blue (clone DX2; Invitrogen, Carlsbad, CA). The levels of CD4 T cells in intestinal biopsies are presented as a percentage of total CD3+ T cells.

CFSE dilution assays were performed as described previously (43). Briefly, PBMCs were prestained with CFSE, and ~1 × 10^6 cells were stimulated in 48-well plates in a volume of 600 μl in RPMI containing 10% human serum at 37°C under 5% CO2 for 6 d. Cells were labeled with p27 Alexa Fluor 700 (clone SK1) and anti-human CD4-PerCP (clone SKD2; Beckman Coulter, Brea, CA) and anti-human CD95-Pacific blue (clone DX2; Invitrogen, Carlsbad, CA). The levels of CD4 T cells in intestinal biopsies are presented as a percentage of total CD3+ T cells.

Measurement of binding Ab responses

SIV Env-specific binding Abs were measured with ELISA using tissue culture-produced SIV Env, captured on a Con A-coated plate as described previously (39). Briefly, ELISA plates (Costar, Cambridge, MA) were coated with Con A (25 μg per well) overnight at 4°C. Plates were washed and incubated with 100 μl undiluted virus-like particle supernatant (generated by transient transfection of 293T cells with the earlier-described SIV239 DNA vaccine expressing Gag, Pol, and Env) for 1 h. Plates were washed and blocked for 1 h (PBS–Tween with 4% w/v and 5% dry milk). Test sera were added to duplicate wells in serial 3-fold dilutions and incubated for 1 h. Plates were then washed, and bound Ab was detected using peroxidase-conjugated anti-mouse IgG (Accurate Chemical and Scientific, Westbury, NY) and tetramethylbenzidine substrate (KPL, Gaithersburg, MD), and reaction was stopped using 2 M sulfuric acid. Absorbances were read at 492 nm in a microplate reader. Measurement of avidity

Downloaded from http://www.jimmunol.org/ by guest on April 14, 2017
100 μg 0.5 μg/ml goat anti-monkey IgA (Rockland, Gilbertsville, PA). 1 μg/ml SIVmac251 rgp130 (ImmunoDiagnostics, Woburn, MA), or 1400 SIVmac251 viral lysate (Advanced Biotechnologies, Columbia, MD), which lacks detectable envelope protein at this dilution. These ELISAs and the serum standards have been described previously (39). Plates were developed by consecutive treatments with biotinylated goat anti-monkey IgA (a Diagnostic, San Antonio, TX) or biotinylated goat anti-human IgG (SouthernBiotech, Birmingham, AL), avidin-peroxidase, tetramethylbenzidine, and 2 N H2SO4. For indirect sections, the concentration of anti-env or anti-gag pol IgA was divided by the total IgA concentration to obtain the sp. act. A secretion was considered IgA Ab-positive if the env or gag pol sp. act. was greater than or equal to 0.145 or 0.224, respectively. These cutoffs represent the mean sp. act. ± 3 SD previously established for rectal secretions from naive macaques.

**Measurement of neutralizing Abs**

SIV-specific neutralization was measured as a function of reductions in luciferase reporter gene expression after a single round of infection in TZM-
bl cells as described (45). TZM-bl cells were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program as contributed by John Kappes and Xiaoyun Wu.

VV neutralization assays were performed as described previously (46). Briefly, 2-fold serial dilutions of sera were incubated with VV-expressing enhanced GFP for 1 h at 37˚C. HeLa S3 cells were added and incubated overnight in the presence of cytosine arabinoside. Fluorescent cells were enumerated with a FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star). ICS values were determined with PRISM software (GraphPad, La Jolla, CA).

**Quantitation of SIV RNA plasma load**

The SIV copy number was determined using a quantitative real-time PCR as previously described (2). All specimens were extracted and amplified in duplicate, with the mean results reported. For viral load determinations in gut, total RNA was extracted from about 1 million cells obtained from gut biopsies and used for quantitative real-time PCR analyses.

**Statistical analysis**

Wilcoxon-Mann-Whitney U test was used for comparisons of T cell responses, Ab responses, and viral RNA levels between Dryvax-immune and Dryvax-naive groups. This method was used because data did not meet with parametric assumptions. The p values were given before correcting for any multiple comparisons. Pearson’s product moment correlation method was used for correlation analysis when data met with parametric assumptions. Spearman’s rank correlation method was used for non-parametric data correlations (indicated as r values on graphs of various figures of this article). A two-sided p < 0.05 was considered significant. Statistical analyses were performed using TIBCO Spotfire S-PLUS 8.1 (TIBCO, Sommerville, MA).

**Results**

We vaccinated two groups of eight macaques with our DNA/MVA SIV239 vaccine that expresses Gag, Pol, and Env and produces noninfectious virus-like particles. The vaccination regimen consisted of i.m. priming with DNA at weeks 0 and 8 and i.m. boosting with MVA at weeks 16 and 24. One of the two vaccinated groups received a single inoculation of Dryvax smallpox vaccine 17 μg before the first DNA prime (Dryvax-immune group). Another eight unvaccinated macaques served as a control group. In each group, four macaques expressed the Mamu A*01 histocompatibility molecule that allowed us to use MHC class I tetramer complexes to follow CD8 T cell responses to the immunodominant Gag epitope CM9 (47). Macaques RLk7 and RGd8 of the Dryvax-naive group were positive for Mamu B08, and the macaque RNv9 of the control group was positive for Mamu B17. All macaques were challenged intrarectally with the highly pathogenic uncloned SIV251 at 9 mo after the final MVA boost. We used uncloned SIV251 rather than SIV239 because the former is a quasi-species.

**Preexisting VV immunity diminishes SIV-specific T cell responses**

As anticipated, prior immunization with Dryvax diminished the magnitude of SIV-specific CD8 and CD4 T cell responses elicited by the DNA/MVA vaccine (Fig. 1A, 1C). We measured the magnitude of SIV Gag-, Pol-, and Env-specific T cell responses in an intracellular cytokine staining assay after the stimulation of PBMCs with peptide pools. Consistent with our previous reports, SIV-specific immune responses were not detected until after the MVA boost (2, 3, 48–50). At 1 wk after the first MVA boost, SIV-specific CD8 T cells ranged from 0.03 to 1.3% (geometric mean of 0.24%) of total CD8 T cells in the Dryvax-naive group (Fig. 1A). Responses were equally directed against Gag and Env. Responses against Pol were observed only in some animals. The SIV-specific responses were further boosted at 1 wk after the second MVA boost to a GM of 0.3% of total CD8 T cells. In contrast, in the Dryvax-immune group, SIV-specific CD8 T cells were boosted by the first but not the second MVA and were 2.4-fold lower (GM of 0.1%) (p = NS) and 12-fold lower (GM of 0.03%) (p = 0.005) than in the Dryvax-naive group at 1 wk after the first and second MVA boosts, respectively (Fig. 1A). This was true for responses directed against Gag or Env (data not shown). Similar patterns were observed for the magnitude of Gag-CM9 tetramer+ cells in Mamu A*01+ animals (Supplemental Fig. 1A).

CFSE proliferation assays on PBMCs prior to challenge also revealed significantly lower Gag-specific CD8 T cell responses in the Dryvax-immune than Dryvax-naive group (Supplemental Fig. 1B).

The magnitude of the SIV-specific CD4 response was also lower in the Dryvax-immune group than that in the Dryvax-naive group (Fig. 1C). In the Dryvax-naive group, the SIV-specific CD4 T cell responses ranged from 0.2 to 1.6% (GM of 0.8%) of total CD4 T cells at 1 wk after the first MVA boost (Fig. 1C) and underwent only a minimal boost after the second MVA immunization. In the Dryvax-immune group, these responses were 2.5-fold lower (GM of 0.3%) (p = 0.03) and 6-fold lower (GM of 0.04%) (p = 0.006) at 1 wk after the first and second MVA boosts, respectively. CFSE proliferation assays on PBMCs prior to challenge also revealed lower Gag-specific CD4 T cell responses in the Dryvax-immune group than those in the Dryvax-naive group (Supplemental Fig. 1B). The magnitude of CD4 T cells that coproduced IFN-γ, TNF-α, and IL-2 was also higher in the Dryvax-naive animals than that in the Dryvax-immune animals (Supplemental Fig. 2B). However, the proportion of IFN-γ, TNF-α, and IL-2 coproducing cells as a percentage of total cytokine-positive cells was similar between the two groups (Supplemental Fig. 2C). A similar pattern was observed for SIV-specific CD8 T cell responses (data not shown).

In contrast with the SIV-specific T cell responses, VV-specific CD8 T cell responses were higher in the Dryvax-immune animals than those in the Dryvax-naive animals. Vaccination with Dryvax elicited a robust VV-specific cellular immune response (Fig. 1B, 1D). After the MVA boosts, both VV-specific CD8 and CD4 T cell responses had ~10-fold higher peaks in the Dryvax-immune animals than those in the Dryvax-naive animals. To understand the influence of preexisting VV immunity on SIV-specific CD8 T cell response, we performed correlations between the magnitude of VV-specific CD8 T cells or VV-specific neutralizing Ab after the first MVA boost and SIV-specific CD8 T cells after the second MVA boost. We chose to look at the effect of preexisting VV immunity at the time of the second MVA boost because this allowed us to look at all animals (Dryvax-immune and Dryvax-naive) at a time when the maximum effect of preexisting immunity would be observed. VV-specific neutralizing Ab as well as VV-specific CD8 T cells prior to the second MVA boost showed inverse correlations with SIV-specific CD8 T cells after the second MVA boost suggesting that both of these responses contributed to the diminished immunogenicity of MVA/SIV vaccine (Supplemental Fig. 3).
Preexisting VV immunity does not diminish SIV-specific humoral immunity

In contrast with the SIV-specific T cell responses, the preexisting VV immunity did not diminish SIV-specific Ab responses (Fig. 2). To evaluate the effect of preexisting VV immunity on the SIV-specific humoral immunity, we measured the titers of binding Ab specific to SIV Env and Gag-Pol and neutralizing Ab to SIV251 in serum. In addition, we determined the avidity index of anti-Env binding Ab in serum. Consistent with our previous reports, SIV Env-specific Ab responses were not detected until after the MVA boost (2, 3, 48–50). At 7 wk after the first MVA boost, the titers of SIV Env-specific binding Ab ranged from 3 to 10 μg/ml (mean of 5 μg/ml) of serum in the Dryvax-naive group (Fig. 2A). These were further boosted to a mean of 108 μg/ml at 1 wk after the second MVA boost. Throughout the period of vaccination, similar titers of Env-specific binding Ab were found in the Dryvax-immune and Dryvax-naive groups. Consistent with the titers of Env-specific binding Ab, at the peak vaccine response (1 wk after second MVA boost), the titers of Gag–Pol–specific binding Ab (Fig. 2B), neutralizing Ab against T cell line-adapted SIV251 (Fig. 2C), and the avidity of Env-specific binding Ab (Fig. 2D) were also similar in the two vaccine groups. Neither group had neutralizing activity for non-T cell line-adapted SIV251 (data not shown).

Non-T cell line-adapted SIV251 was also present in rectal secretions of four of the eight Dryvax-immune animals. This IgA was found in only one of the eight Dryvax-naive animals and none of the unvaccinated controls suggesting that preexisting VV immunity had promoted the generation of long-lasting SIV-specific rectal IgA.

As expected, MVA-specific neutralizing Ab responses were higher in the Dryvax-immune animals than those in the Dryvax-naive animals (Fig. 2F). These responses were 100-fold higher in the Dryvax-immune animals than those in the Dryvax-naive animals after the first MVA boost and ~2-fold higher after the second MVA boost. Analyses for VV-specific neutralizing Ab activity showed a similar pattern (data not shown).

Preexisting VV immunity does not diminish the control of a pathogenic intrarectal SIV challenge

Despite their low SIV-specific T cell responses, the Dryvax-immune macaques exhibited the best control of an intrarectal SIV challenge (Fig. 3 and Supplemental Fig. 5). After SIV challenge, viremia was detected in all animals except RLr8 of the Dryvax-immune group (Fig. 3A). In RLs8, a low level of viremia was detected, but only at 2 wk postchallenge (Fig. 3A). We observed a 5- to 60-fold increase in the frequency of proliferating (Ki-67+; CFSElo) Gag-specific CD4 and CD8 T cells at 2 wk postchallenge in RLr8 and RLs8.
suggesting both animals had been infected (Fig. 4A, 4B). In addition, the titer of serum anti-Env binding Ab increased 3-fold in RLr8 and 1.5-fold in RLs8 at 2 wk postchallenge (Fig. 4C).

At 2 wk postchallenge, plasma viral RNA in the control animals ranged from $6 \times 10^5$ to $2.6 \times 10^8$ copies per milliliter with median copies of $2.0 \times 10^7$/milliliter. At this time, the median levels of plasma viral RNA were 10 times lower in the Dryvax-naive animals ($p = 0.005$) and 150 times lower in the Dryvax-immune animals ($p = 0.01$) than those in the unvaccinated control animals (Fig. 3A, 3B). The median levels of plasma viral RNA in Dryvax-immune animals were 15 times lower than those in the Dryvax-naive animals (Fig. 3A, 3B). Viral control during set point was also lower in the Dryvax-immune animals than that in control animals. At 24 wk, the Dryvax-immune animals had levels of virus that were six times lower than those in the Dryvax-naive animals ($p = 0.03$) and 17 times lower than those in the unvaccinated controls (Supplemental Fig. 4C, 4D). Furthermore, none of the Dryvax-immune animals were positive for Mamu B08 and B17 indicating that the enhanced control was not due to expression of known protective Mamu class I alleles.

Consistent with the lower levels of virus, depletion of colorectal CD4 T cells was slower and less severe in the Dryvax-immune animals than that in the Dryvax-naive and the control animals (Fig. 3C, 3D). In the majority of the Dryvax-immune animals, the nadir of CD4 T cells had occurred by 3 wk postchallenge, whereas in the majority of animals in the other two groups, the nadir of CD4 T cells did not occur until 6 wk postchallenge (Fig. 3C, 3D). Furthermore, in four of the eight Dryvax-immune animals, colorectal CD4 T cells were rebounding by 24 wk. At 24 wk postchallenge, the frequencies of central memory CD4 T cells in the blood ($p = 0.03$) were higher in the vaccinated animals than those in the control animals (Supplemental Fig. 5A).

Enhanced viral control correlates with lower colorectal virus early postchallenge

A closer look at the kinetics of viremia during the first 3 wk of infection revealed slow expansion of viremia in five Dryvax-immune animals and one Dryvax-naive animal (Fig. 3A, 3B). In
these six animals, virus reached peak levels at ≥3 wk, whereas in the remaining vaccinated and control animals, virus reached peak levels at 2 wk postchallenge (Fig. 3F). The slower expansion of viremia resulted in lower peak and set point viremia. Animals in which virus peaked at ≥3 wk had a 243-fold lower median peak viremia and a 159-fold lower median set point viremia (week 24) (Fig. 3E).

Impressively, the levels of colorectal virus at 2 wk postchallenge influenced the kinetics and magnitude of viremia. The median level of colorectal virus was 150-fold lower in animals in which peak viremia was delayed to ≥3 wk (Fig. 3F). All animals in which viremia peaked at week 2 had levels of colorectal virus above 10⁴ copies/500 ng total tissue RNA, whereas all animals in which viremia peaked at or after week 3 had colorectal virus below this level. The levels of colorectal virus in five of the Dryvax-immune animals and one of the Dryvax-naive animals were below this level, whereas in all of the remaining vaccinated and control animals, colorectal virus was above this level (Fig. 3F). The median level of colorectal virus in Dryvax-immune animals was 39-fold and 49-fold lower than that in Dryvax-naive (p = 0.02) and control (p = 0.02) animals, respectively. Furthermore, the levels of colorectal virus showed a strong direct correlation with peak (week 2; p = 0.006) and set point (week 24; p = 0.02) viremia (Fig. 3G). In addition, an inverse correlation was observed between the frequencies of colorectal virus and blood at acute (week 2 or 3) and set point (weeks 24) phases after challenge. In addition, we measured the frequency of Gag CM9-specific CD8 T cells in blood and colorectal tissue. However, the tetramer analyses were restricted to Mamu A*01* animals. At 2 wk after challenge, expansion of Gag-CM9–specific T cells was higher in colorectal tissue (Fig. 5A) and blood (Fig. 5C) of vaccinated animals than that in unvaccinated animals. In general, in vaccinated animals, expansion was higher in animals with higher levels of virus suggesting that higher expansion was in response to higher levels of Ag (Fig. 5B, 5D). Analyses for SIV-specific IFN-γ–producing CD8 T cells in blood revealed similar patterns (Fig. 5E, 5F). Correlations between SIV-specific CD8 T cells at 1 wk after the second MVA boost and viral load at 2 wk after challenge in colorectal tissue or plasma also revealed a direct correlation (data not shown). Consistent with the variability in viremia in the Dryvax-immune group (Fig. 3B), the frequencies of SIV-specific CD8 T cells postchallenge showed the most variability in this group (Fig. 5A, 5C, 5E). In some of the Dryvax-immune animals, high levels of expansion were observed despite these animals having low levels of CD8 responses after vaccination (Fig. 5E).

Non-CD8 T cell correlates for reduced levels of colorectal virus

We next investigated the relationship between vaccine-elicited Ab responses, CD4 T cell responses, and colorectal virus (Fig. 6). These analyses suggested significant roles for SIV-specific IgA in rectal secretions, the avidity of serum binding Ab to the native form of Env, and the magnitude of vaccine-elicited SIV-specific CD4 T cells in the early control of colorectal virus. Animals that were positive for antiviral IgA in rectal secretions prior to challenge had 96-fold lower levels of virus in colorectal tissue at 2 wk postchallenge than those in animals that were negative for IgA (Fig. 6A). At 2 wk after challenge, the SIV-specific IgA responses were present in rectal secretions of all animals that were positive prior to challenge, except for RLr8, which had levels of virus below detection (Table I). At this time, three additional animals in the Dryvax-naive group, one additional animal in the Dryvax-immune group, and the only spontaneous controller in the unvaccinated control group (RBy9) had developed SIV-specific IgA responses in rectal secretions.

The avidity of vaccine-elicited Ab for the native form of Env also showed an inverse correlation with viral RNA levels at 2 wk after challenge both in colorectal tissue (p = 0.03) and blood (p = 0.013) (Fig. 6B, 6C). As expected, the avidity indices were higher postchallenge than postvaccination. A direct correlation was observed for avidity indices postvaccination and postchallenge suggesting that animals that had higher-avidity Ab postvaccination also had higher-avidity Ab postchallenge (Fig. 6D). In contrast with the avidity indices where there was a correlation with reduced viremia, the titers of anti-Env binding Ab or neutralizing Ab against T cell line-adapted SIV251 showed direct correlations with plasma viral load suggesting the expansion of these responses to higher levels of viral Ag (p < 0.01; data not shown). None of the vaccinated and control animals developed neutralizing activity for non-T cell line-adapted SIV251 until 12 wk postchallenge (data not shown).

The frequency of IFN-γ–secreting virus-specific CD4 T cells at 1 wk after the second MVA boost showed a direct correlation with levels of viral RNA in colorectal tissue at 2 wk after challenge (Fig. 6E) (p = 0.03). A direct correlation was also observed between the frequency of Gag-specific proliferating CD4 T cells at 4
FIGURE 3. Preexisting VV immunity does not diminish the control of a pathogenic intrarectal SIV challenge. A and B, Temporal viremia median (A) for the group and (B) for individual animals. C and D, Frequency of CD4 T cells expressed as a percentage of total T cells in the colorectal tissue median (C) for group and (D) for individual animals. Dotted lines indicate Mamu A*01+ animals, and solid lines indicate Mamu A*01- animals. E, Median temporal levels of plasma viral RNA in animals that peaked at week 2 and at or after week 3. F, Comparison of colorectal virus at 2 wk postchallenge by time of peak in plasma. Key to animal names is presented in Fig. 3B. Boxes represent medians with 25th and 75th percentiles for the group. G, Correlation between levels of viral RNA in the rectum at week 2 and plasma at weeks 2 and 24. Dryvax-naive animals are shown by closed triangles (n = 8), Dryvax-immune animals by closed squares (n = 8), and controls by open triangles (n = 8). The sensitivity of viral load assay was 80 copies of RNA/ml, and animals with levels of virus below 80 were scored at 100. r_s, Spearman’s rank correlation.
wk prior to challenge and colorectal virus at 2 wk after challenge (Fig. 6E) \((p = 0.04)\). Furthermore, the frequencies of virus-specific CD4 T cells displaying the CCR5 viral coreceptor were 3-fold higher \((p = 0.01)\) after the first MVA boost and 7-fold higher \((p = 0.005)\) after the second MVA boost in the Dryvax-naive animals (Fig. 6F) demonstrating that preexisting VV immunity had diminished the elicitation of CCR5+ CD4 T cells.

**Discussion**

Our study evaluating the effect of preexisting VV immunity on cellular and humoral immunity elicited by a DNA/MVA SIV vaccine in rhesus macaques demonstrates that preexisting immunity reduces the magnitude of SIV-specific cellular but not humoral immunity. This was true for both IgG as well as IgA responses in serum. To our knowledge, this is the first report demonstrating that preexisting poxvirus immunity does not influence the elicitation of humoral immunity after an i.m. immunization with a poxvirus vector. These results strongly suggest that preexisting VV immunity may not be a limitation for the induction of Ab responses by recombinant MVA vaccines.

The mechanisms that contributed to the inability of preexisting VV immunity to diminish MVA insert-specific humoral immunity are not clear and occurred despite the presence of high titers of MVA-specific neutralizing Ab and T cell responses at the time of MVA boosts. Previous studies have shown that a relatively small amount of Ag is needed for inducing a strong humoral immune response if the Ag is presented in the form of immune complexes \((52–54)\). We speculate that immune complexes formed between preexisting SIV-specific Ab prior to the boost and low levels of SIV Ag present after the boost contributed to the observed strong boost of SIV-specific humoral immunity in Dryvax-immune animals. If this is true, similar mechanisms may be applicable for other viral vectors.

A critical finding of our study is that preexisting VV immunity does not diminish the ability of a DNA/MVA SIV vaccine to control a pathogenic intrarectal SIV challenge despite reducing the magnitude of SIV-specific cellular immunity. In fact, four of the eight Dryvax-immune animals controlled the virus below 1000 copies/ml of plasma. This control was not due to the presence of known protective Mamu class I alleles. However, we did observe strong antiviral CD8 T cell responses postchallenge in the Dryvax-immune animals.
immune animals despite the low frequencies of these cells post-vaccination. These results suggest that preexisting immunity could have influenced the functional quality of SIV-specific CD8 T cells elicited by the DNA/MVA vaccine such that they possess enhanced expansion potential.

In our study, the level of virus replication in colorectal tissue at 2 wk postchallenge correlated with the tempo of infection in blood. Levels of colorectal virus below $10^4$ copies/500 ng tissue RNA were associated with viremia that peaked at ≥3 wk, whereas levels greater than this were associated with viremia that peaked at 2 wk. Impressively, the magnitude of peak viremia was 248-fold lower in animals in which viremia peaked at ≥3 wk than that in animals in which viremia peaked at 2 wk. These results demonstrate that early control of virus replication at the colorectal site of

**FIGURE 5.** No clear association between the SIV-specific CD8 T cell response and viremia in colorectal tissue early after infection. A, Temporal SIV Gag-CM9–specific CD8 T cell responses in the colorectal tissue of Mamu A*01* animals. B, Correlation between tetramer-specific CD8 T cells and levels of virus in the colorectal tissue. C, Temporal SIV Gag-CM9–specific CD8 T cell responses in blood of Mamu A*01* animals. D, Correlation between tetramer-specific CD8 T cells and levels of virus in blood. E, SIV (Gag and Env)-specific IFN-γ–producing CD8 T cells in blood. F, Correlation between SIV-specific CD8 T cells and levels of virus in blood. Key to animal names is presented in Fig. 3B. C, control; DI, Dryvax-immune; DN, Dryvax-naive.
challenge correlates with protection and highlight the importance of immunodeficiency virus vaccines achieving early control at mucosal sites of challenge.

Our results suggest that immune mechanisms that block virus infection and reduce the frequency of target cells in colorectal tissue limit virus replication at the site of infection. We identified three factors—antiviral IgA in prechallenge rectal secretions, the avidity of anti-Env IgG, and the level of vaccine-elicited CD4 T cells bearing the CCR5 viral coreceptor—that influenced the magnitude of colorectal virus early after challenge. The presence prechallenge of antiviral IgA in rectal secretions was associated with enhanced control of colorectal virus. The antiviral IgA in rectal secretions in our assays was directed against Gag-Pol and not Env. Anti–Gag-Pol IgA has been shown to neutralize HIV-1 replication inside epithelial cells (55). More importantly, the anti–Gag-Pol activity may have served as an indicator of protective functions that we do not understand. Consistent with our previous studies with SHIV challenges (39, 56), the avidity of anti-Env IgG, and the level of vaccine-elicited CD4 T cells bearing the CCR5 viral coreceptor—that influenced the magnitude of colorectal virus early after challenge. The presence prechallenge of antiviral IgA in rectal secretions was associated with enhanced control of colorectal virus. The antiviral IgA in rectal secretions in our assays was directed against Gag-Pol and not Env. Anti–Gag-Pol IgA has been shown to neutralize HIV-1 replication inside epithelial cells (55). More importantly, the anti–Gag-Pol activity may have served as an indicator of protective functions that we do not understand. Consistent with our previous studies with SHIV challenges (39, 56), the avidity of anti-Env IgG,

FIGURE 6. Non-CD8 T cell-mediated correlates for enhanced colorectal viral control. A, Comparison of levels of virus in the colorectal tissue and blood based on IgA positivity in rectal secretions prior to challenge. B and C, Correlation between avidity indices after vaccination and viral load (B) in the rectum and (C) in blood. D, Correlation between avidity indices after vaccination and challenge. E, Correlation between SIV-specific IFN-γ-secreting CD4 T cells at 1 wk after the second MVA boost or SIV Gag-specific proliferating CD4 T cells 4 wk prior to SIV challenge and levels of colorectal virus at 2 wk postchallenge. F, Frequency of SIV Gag-specific CCR5+ CD4 T cells. On the left, representative FACS plots. Cells were gated on CD3+ and CD4+. Numbers on the graphs represent the frequency of CCR5+ cells as a percentage of total CD4 T cells. On the right, summary of CCR5+ CD4 T cells after first and second MVA boosts. Boxes represent medians with 25th and 75th percentiles for the group. Key to animal names is presented in Fig. 3B. DI, Dryvax-immune; DN, Dryvax-naive; \( r_s \), Spearman’s rank correlation.

Ab-dependent cell-mediated virus infection activities that in turn correlate with enhanced control of an SHIV challenge (57). In the same study, a direct correlation was also observed between the magnitude of HIV Env-specific IgA in rectal secretions and inhibition of transcytosis activity of HIV in vitro. Thus, we speculate that high-avidity Ab in our study may be working through ADCC and ADCVI activities and the rectal IgA through inhibition of transcytosis activity.

In our study, low levels of vaccine-elicited CD4 T cells showed a moderate correlation with enhanced control of colorectal virus. Although the role of vaccine-elicited CD4 T cells in the initiation of HIV/SIV infection during a mucosal challenge remains to be delineated, there is growing evidence suggesting that these cells preferentially support virus replication (58, 59). Mattapallil et al. (60) demonstrated a preferential infection of virus-specific CD4 T cells in the blood of vaccinated but not unvaccinated animals at day 7 after SIV challenge. In the future, it is important to measure the frequency of virus-specific CD4 T cells in the blood of vaccinated but not unvaccinated animals at day 7 after SIV challenge. In the future, it is important to measure the frequency of virus-specific CD4 T cells in the blood of vaccinated but not unvaccinated animals at day 7 after SIV challenge. In the future, it is important to measure the frequency of virus-specific CD4 T cells in the blood of vaccinated but not unvaccinated animals at day 7 after SIV challenge.
Env Ab. These animals also had low levels of vaccine-induced CD4 T cells. One of the two (RLs8) showed definitive signs of infection including a low level of viremia at 3 wk postchallenge and a transient depletion of colorectal CD4 T cells and anamnestic antiviral IgG and IgA responses postchallenge. The second (RLr8) showed a transient 3-fold expansion of anamnestic antiviral IgG in serum at 2 wk postchallenge but no detectable levels of viremia. Both animals showed strong anamnestic Gag-specific CD8 and CD4 T cell responses at 2 wk postchallenge. We consider this as potential sterilizing immunity mediated by a combination of high-avidity binding Ab, rectal IgA, and the presence of low levels of antiviral CD4 T cell targets for infection.

In conclusion, our results show that preexisting VV immunity does not diminish the control of a pathogenic intrarectal immunodeficiency virus challenge by a DNA/MVA vaccine despite it reducing antiviral cellular but not humoral responses. Our results show that immune mechanisms mediating early viral control in colorectal tissue enhance control of both acute and chronic phases of immunodeficiency virus infections and highlight the critical need for controlling virus replication at the site of viral challenge. And finally, our results show two vaccine-elicited Ab responses, antiviral mucosal IgA and high-avidity anti-Env IgG, contributing to early mucosal viral control.

Acknowledgments

We thank Dr. J. Altman for provision of Gag-CM9 tetramer, J. Amico for preparation of rMV A vaccines and for MV A neutralization assays, and Helen Drake-Perrow for outstanding administrative support. We thank Dr. F. Villinger for critical reading of the manuscript and Dr. D. Watkins and the Wisconsin National Primate Research Center Genotyping Service (Madison, WI) for help with Mamu B08 and B17 typing of animals. We also thank the Yorkes Division of Research Resources (Atlanta, GA) for the consistently excellent pathology support. We thank the Emory Center for AIDS Research virology core (Atlanta, GA) for viral load assays. Also, we thank the National Institutes of Health AIDS Research and Reference Reagent Program (Germantown, MD) for the provision of peptides.

Disclosures

H.L.R. is a cofounder and holder of equity in Geovax Labs, Inc.

References

**Supplementary Figure 1.** Gag-specific T cell responses post vaccination. (A) Gag CM9 tetramer-specific CD8 T cells following vaccination. (B) Gag-specific proliferative CD4 and CD8 T cell responses in blood 4 weeks prior to SIV challenge measured using CFSE dilution assay. Key to animal names is presented in Figure 3B.

**Supplementary Figure 2.** Cytokine co-expression profiles of SIV Gag-specific CD4 T cells at one week post the 1st MVA boost. (A) Schematic for the analysis of cytokine co-expression profiles of CD4T cells following stimulation with Gag peptide pool. Lymphocytes were gated based on forward and side scatter and CD4 T cells (CD3+, CD8-, CD4+) were then analyzed for IFN-γ, IL-2 and TNF-α expression. Cytokine co-expression profiles were determined using Boolean gating function of FlowJo software. (B) Frequency of triple (TP), double (DP) and single (SP) producers expressed as a percent of total CD4 T cells. Bars represent mean ± SEM (n=8 for each group). (C) Pie chart represent the quality of the response with the subsets (TP, DP and SP) expressed as percent of total cytokine positive CD4 T cells (n=8 for each group). I, IFNγ; L, IL-2 and T, TNFα.

**Supplementary Figure 3.** VV-specific humoral and cellular immunity contributes to diminished SIV-specific CD8 T cell response. Correlation between VV-specific CD8 T cell or neutralizing antibody responses prior to 2nd MVA boost and the magnitude of SIV-specific CD8 T cell responses at one week following the 2nd MVA boost. Each symbol represents an individual macaque.
**Supplementary Figure 4. Analysis of plasma viral load by A*01 status.** Temporal Plasma viral RNA (A) median for the group and (B) for individual animals in Mamu A*01+ animals. Temporal Plasma viral RNA (C) median for the group and (D) for individual animals in Mamu A*01- animals. The sensitivity of viral load assay was 80 copies of RNA/ml, and animals with levels of virus below 80 were scored at 100. Viral loads from an additional 5 unvaccinated rhesus macaques (2 Mamu A*01 and 3 non-Mamu A*01) challenged intrarectally with the same virus and dose(50) were included for these subgroup analyses. ‘*’ denote significant differences from control animals at the indicated timepoints.

**Supplementary Figure 5. Correlation between colorectal virus and depletion of gut CD4 T cells post challenge.** (A) Frequency of total central memory (CD28+ CD95+) CD4 T cells in blood at 24 weeks following SIV251 challenge. Correlation between colorectal virus and percent CD4 T cells in colorectal tissue at (B) week 3 and (C) week 24 post challenge. DN, Dryvax-naïve; DI, Dryvax-immune; C, Controls. r_s, Spearman’s rank correlation.
Suppl 1

A

% Gag-CM9 +ve CD8 T cells (Blood)

Pre-DNA (wk2)
2nd DNA
1st MVA
2nd MVA

p = 0.003

B

% CFSE-ve Ki67+ CD4 - T cells (Blood)

p = 0.008

% CFSE-ve Ki67+ CD8 - T cells (Blood)

p = 0.004

DN - Dryvax -naive
DI - Dryvax -immune
C - Controls
Suppl 3

A

- **VV-specific CD8 T cells** (week 1, 1st MVA)
  - $r = 0.61$
  - $p = 0.01$

- **SIV-specific CD8 T cells** (week 1, 2nd MVA)
  - $r = 0.76$
  - $p = 0.0006$

Legend:
- ▲ Dryvax-Naive
- ▼ Dryvax-Immune
Suppl 4

**A**
- ▲ Dryvax-Naive
- ■ Dryvax-Immune
- ▼ Controls

**B**

**Dryvax-Naive**
- ▲ ROw-7
- ▼ REk-7
- ▼ RNI-7
- ▼ RGe-8

**Dryvax-Immune**
- ▲ RGr-8
- ▼ RVp-8
- ▼ RLS-8
- ▼ RKn-8

**Controls**
- ▲ RPw-9
- ▼ RQr-9
- ▼ RRk-10
- ▼ RYd-9
- ▲ RAr-6
- ▼ RVo-6

**C**
- ▲ Dryvax-Naive
- □ Dryvax-Immune
- ▼ Controls

**D**

**Dryvax-Naive**
- ▲ REp-7
- ▼ RHs-7
- ▼ RN-8
- ▼ RTm-7

**Dryvax-Immune**
- ▲ RBS-8
- ▼ RR-8
- ▼ ROb-8
- ▼ ROk-8

**Controls**
- ▲ RAc-10
- ▼ RBy-9
- ▼ RNv-9
- ▼ RFp-9
- ▲ ROk-6
- ▼ RRR-6
- ▼ RKn-6

**A*01**

**Non A*01**