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

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The influence of preexisting immunity to viral vectors is a major issue for the development of viral-vectored 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 vaccines achieving early control at the mucosal sites of challenge. ***The Journal of Immunology*****, 2010, 185: 7262–7273.**

Live 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-vectored 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

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Abbreviations used in this paper: Ad5, adenovirus type 5; C, control; DI, Dryvax-immune; DN, Dryvax-naive; GM, geometric mean; MVA, modified vaccinia Ankara; r_s, Spearman's rank correlation; SHIV, simian HIV; TCLA, T cell line adapted; VV, vaccinia virus.

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(35) has successfully completed phase I safety testing and entered phase II trials in humans in the United States. The preexisting anti-VV immunity generated by the smallpox vaccine may limit the boosting ability of rMVA and hence the efficacy of DNA/MVA vaccines. In this study, we evaluated the effect of preexisting VV immunity on the immunogenicity and efficacy of a DNA/MVA SIV vaccine in rhesus macaques using a high-dose pathogenic intrarectal SIV251 challenge. Our results demonstrate that preexisting immunity diminishes cellular but not humoral immunity. They also demonstrate that diminished cellular immunity does not reduce the efficacy of the DNA/MVA vaccine and suggest a role for nonneutralizing antiviral Ab in viral control.

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 rMVA 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 rMVA boosters were given on weeks 16 and 24. The DNA was delivered at 1.2 mg/dose, and the rMVA was delivered at 1×10^8 PFU/dose. Of the 16 vaccinated animals, 8 received standard dose of Dryvax (Wyeth Laboratories, Madison, NJ) ($\sim 10^5$ PFU) by percutaneous route 17 mo prior to first DNA inoculation. At 9 mo after the final rMVA 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 μl of the stock diluted in 1 ml RPMI 1640 was used per animal. One hundred percent of animals can be infected under these conditions.

Collection and processing of rectal secretions, biopsies, and blood

Rectal secretions were collected with and eluted from Weck-Cel sponges as previously described (39, 42). PBMCs were isolated from whole blood according to the standard procedures as described previously (33). Lymphocytes from pinch biopsies from the rectum were obtained as described previously (43). Briefly, 10 to 20 pinch biopsies were collected in complete RPMI 1640 and washed two times with ice-cold HBSS. Biopsies were digested with 200 U/ml collagenase IV (Worthington, Lakewood, NJ) and DNase I (Roche, Indianapolis, IN), passed through decreasing sizes of needles (16-, 18-, and 20-gauge, five to six times with each needle), and filtered through a 100-μm filter. Cells were washed twice with RPMI and resuspended in complete RPMI for analysis.

T cell responses

Intracellular cytokine production was assessed as previously described with a few modifications (2). Briefly, 2 million PBMCs were stimulated in 200 μl RPMI with 10% FBS in a 5-ml polypropylene tube. For measuring vaccinia-specific responses, $\sim 5 \times 10^6$ PFU (multiplicity of infection of ~2.5) of VV strain WR was added in a volume of 100 μl. SIV-specific stimulations were conducted using a single pool of 125 SIV239 Gag peptides, a single pool of 221 SIV239 Env peptides, and 2 pools (132 peptides/pool) of SIV239 Pol peptides (National Institutes of Health AIDS Research and Reference Reagent Program, Germantown, MD). All peptides were 15-mers overlapping by 11. Staphylococcal enterotoxin B was used as a positive control at 1 μg/ml. Stimulations were performed in presence of anti-CD28 and anti-CD49d Abs (1 μg/ml; BD Pharmingen, San Diego, CA). For all stimulations, cells were incubated at 37°C in the presence of 5% CO_2 for 6 h except for stimulations using VV, where stimulations were performed for 10–12 h. Brefeldin A (10 μg/ml) was added for the last 4 h of incubation. At the end of stimulation, cells were washed once with PBS containing 2% FBS, surface stained with anti-human CCR5-PE (clone 3A9; BD Pharmingen), anti-human CCR7-FITC (clone 150503; R&D Systems, Minneapolis, MN), anti-human CD4-PerCP (clone L200; BD Pharmingen), and anti-human CD8-AmCyan (clone SK1; BD Biosciences, San Jose, CA), fixed with Cytofix/Cytoperm (BD Pharmingen), and permeabilized with 1× Permwash (BD Pharmingen). Cells were then stained using a mixture of Abs containing anti-human CD3-Pacific blue (clone SP34-2; BD Pharmingen), anti-human IFN-γ Alexa 700 (clone B27; BD Pharmingen), anti-human IL-2–allophycocyanin (clone MQ1-17H12; BD Pharmingen), and anti-human TNF-α–PE–Cy7 (clone Mab11; eBioscience, San Diego, CA), washed twice with Permwash, once with 2% FBS in PBS, and resuspended in 1% formalin in PBS. Approximately 500,000 lymphocytes were acquired on the LSRII (BD Immunocytometry Systems, San Jose, CA) and analyzed using FlowJo software (Tree Star, Ashland, OR). Lymphocytes were identified based on their scatter pattern, and $CD3^+CD8^-CD4^+$ cells were considered as CD4 T cells, and $CD3^+CD8^+CD4^-$ cells were considered as CD8 T cells. These CD4 or CD8 T cells were then gated for cytokine-positive cells.

T cells were subjected to tetramer staining and typing for the presence of CD4 and CD8 T cells. This was done using a mixture of the following Abs and Gag-CM9 tetramer conjugated to allophycocyanin: anti-human CD3-Alexa Fluor 700 (clone SP34-2; BD Pharmingen), anti-human CD4-PerCP (clone L200, BD Pharmingen), anti-human CD8-AmCyan (clone SK1; BD Biosciences), anti-human CD28-PE-Cy7 (clone CD28.2; 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 $\sim 1 \times 10^6$ cells were stimulated in 48-well plates in a volume of 600μl in RPMI containing 10% human serum at 37°C under 5% CO_2 for 6 d. Cells were stimulated with pooled peptides spanning the entire SIV Gag protein (single pool of 125 peptides, cat no. 6204, National Institutes of Health AIDS Research and Reference Reagent Program) at a concentration of 1.0 μg/ml of each peptide. Unstimulated cells served as negative controls. At the end of 6 d in culture, the cells were stained intracellularly using Abs specific for Ki-67 (clone B56), CD8 (clone SK1), and CD3 (clone SP34-2), and acquired on a FACSCalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star).

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; Corning Life Sciences, Lowell, MA) were coated with Con A (25 μg per ml) 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% whey 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-monkey IgG (Accurate Chemical and Scientific, Westbury, NY) and tetramethylbenzidine substrate (KPL, Gaithersburg, MD). Reactions were stopped with 100 μl 2 N H_2SO_4. Each plate included a standard curve generated using goat anti-monkey IgG and rhesus IgG (both from Accurate Chemical and Scientific Corp.) as previously (39). Standard curves were fitted and sample concentrations interpolated as micrograms of Ab per milliliter of serum using SOFTmax 2.3 software (Molecular Devices, Sunnyvale, CA). The concentrations of IgG are relative to our standard curve, not absolute values.

An NaSCN displacement ELISA assay modeled after that described by Vermont et al. (44) was used for determining avidity. This assay was conducted using parallel titrations of test sera in our standard ELISA assay. After the binding of the test sera, the parallel titrations were treated for 15 min at room temperature with PBS or 1.5 M NaSCN (prepared fresh in PBS). Then, the relative levels of bound Ab were determined using the standard ELISA procedure (see earlier). The avidity index was calculated by dividing the dilution of the serum that gave an OD of 0.5 with NaSCN treatment by the dilution of the serum that gave an OD of 0.5 without NaSCN treatment and multiplying by 100. Each assay included one plate with a standard serum with known avidity. Interassay variation in the avidity index for the standard serum was ±3 for an index of 27.

Measurements for total IgA, anti-SIV env IgA, or anti-SIV gag,pol IgA or IgG were done by ELISA using microtiter plates coated respectively with

100 μl 0.5 μg/ml goat anti-monkey IgA (Rockland, Gilbertsville, PA), 1 μg/ml SIVmac251 rgp130 (ImmunoDiagnostics, Woburn, MA), or 1/400 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 (α Diagnostics, San Antonio, TX) or biotinylated goat anti-human IgG (SouthernBiotech, Birmingham, AL), avidin-peroxidase, tetramethylbenzidine, and 2 N H_2SO_4. For rectal secretions, 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). IC_{50} 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 nonparametric data correlations (indicated as r_s 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 mo 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 histocompatability molecule that allowed us to use MHC class I tetrameric 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. 1*A*, 1*C*). 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 [GM] of 0.24%) of total CD8 T cells in the Dryvax-naive group (Fig. 1*A*). 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. 1*A*). 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. 1*A*). 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. 1*B*).

The magnitude of the SIV-specific CD4 response was also lower in the Dryvax-immune group than that in the Dryvax-naive group (Fig. 1*C*). 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. 1*C*) 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. 1*B*). 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. 2*B*). 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. 2*C*). 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 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. 1*B*, 1*D*). 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).

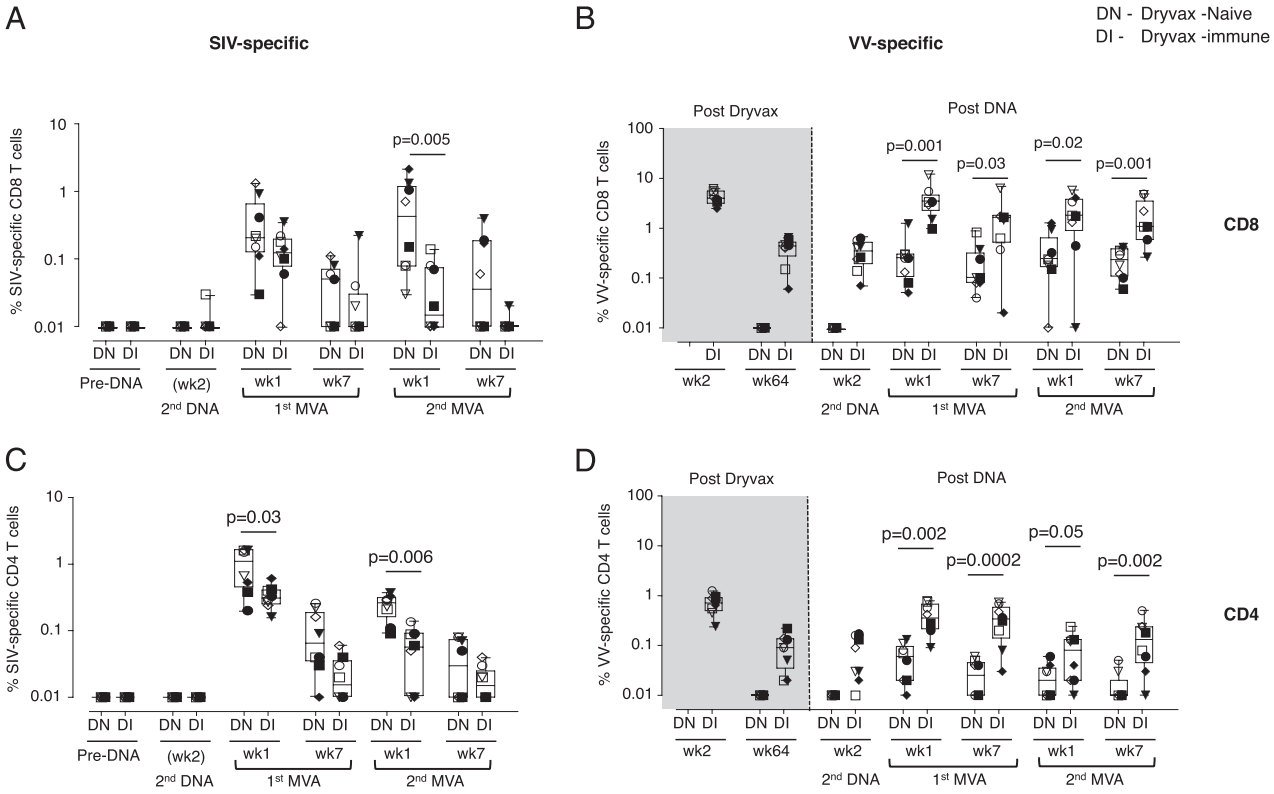

FIGURE 1. Preexisting immunity to vaccinia limits the MVA insert-specific cellular immunity elicited by a DNA/MVA SIV vaccine. *A*, SIV (Gag, Env, and Pol)-specific IFN-γ–producing CD8 T cell responses. *B*, VV-specific IFN-γ–producing CD8 T cell responses. *C*, SIV (Gag, Env, and Pol)-specific IFN-γ–producing CD4 T cell responses. *D*, VV-specific IFN-γ–producing CD4 T cell responses. Closed symbols represent Mamu A*01^{+} animals, and open symbols represent Mamu A*01^{-} animals. Boxes represent medians with 25th and 75th percentiles for the group. Key to animal names is presented in Fig. 3*B*. DI, Dryvax-immune (n = 8); DN, Dryvax-naive (n = 8).

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. 2*A*). 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. 2*B*), neutralizing Ab against T cell line-adapted SIV251 (Fig. 2*C*), and the avidity of Env-specific binding Ab (Fig. 2*D*) were also similar in the two vaccine groups. Neither group had neutralizing activity for non-T cell line-adapted SIV251 (data not shown).

We next investigated the effect of preexisting immunity on SIV-specific IgA responses in serum and rectal secretions after vaccination. Measurements in serum were performed at 1 wk and 9 mo (6 wk prior to challenge) after the second MVA boost. Measurements in rectal secretions were performed prior to challenge. At 1 wk after the second MVA boost, all animals had Env-specific serum IgA (Fig. 2*E*), and one animal in each group had significant levels of Gag-Pol–specific IgA (data not shown). These Env responses were similar between the two vaccine groups demonstrating that preexisting VV immunity did not diminish the SIV-specific IgA response. However, the serum IgA response was transient, and prior to challenge, all animals were negative for Env-specific serum IgA (data not shown). In contrast, 6 wk prior to challenge, SIV Gag-Pol (Table I), but not Env (data not shown), specific IgA was 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. 2*F*). 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. 3*A*). In RLs8, a low level of viremia was detected, but only at 2 wk postchallenge (Fig. 3*A*). We observed a 5- to 60-fold increase in the frequency of proliferating (Ki-67^{+}, CFSE^{lo}) Gag-specific CD4 and CD8 T cells at 2 wk postchallenge in RLr8 and RLs8

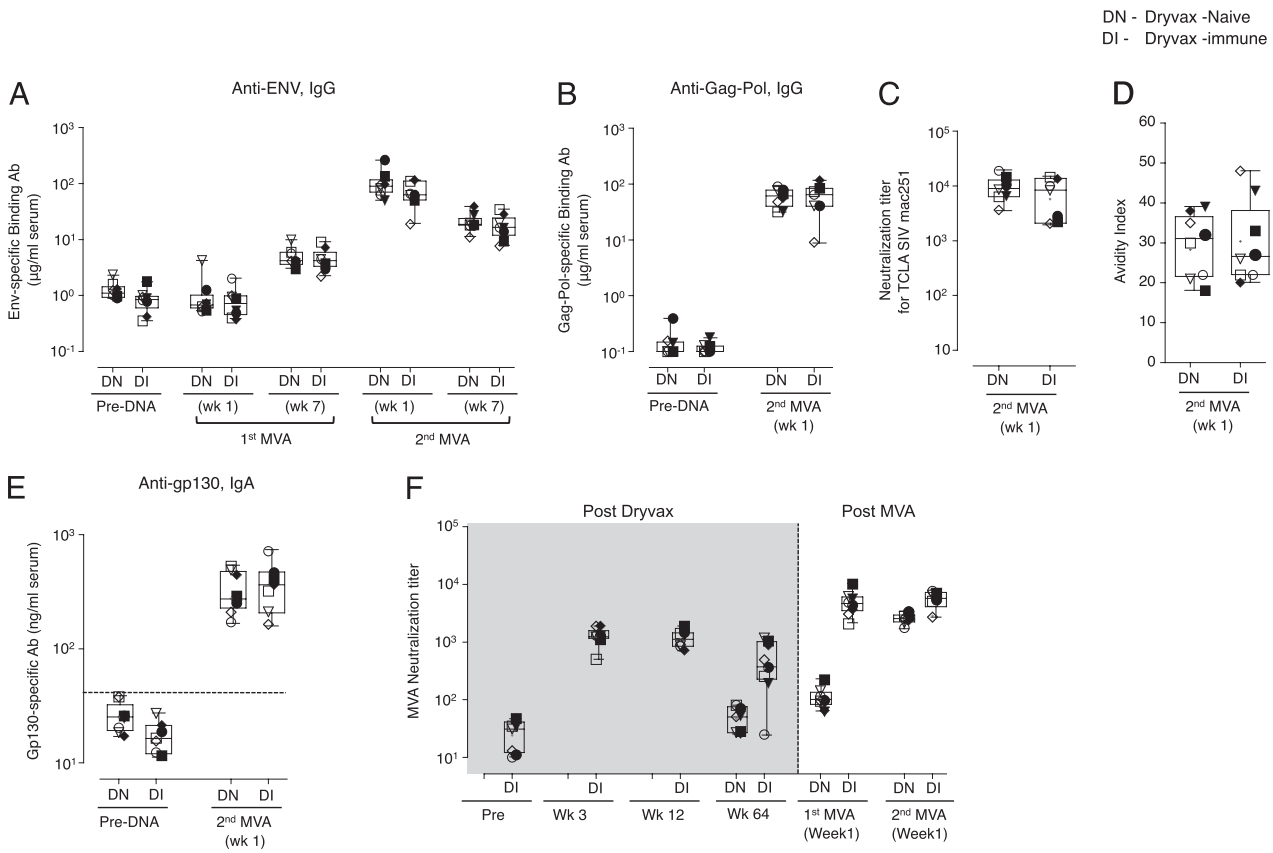

FIGURE 2. Preexisting immunity to vaccinia does not limit the MVA insert-specific humoral immunity elicited by a DNA/MVA SIV vaccine. *A*, SIV Env-specific IgG in serum. *B*, SIV Gag-Pol–specific IgG in serum. *C*, T cell line adapted (TCLA) SIV-specific neutralizing Ab titer in serum. *D*, Avidity index of SIV Env-specific IgG in serum. *E*, SIV Env-specific IgA in serum. The horizontal dashed line indicated the limit of background. *F*, VV-specific neutralizing Ab titer in serum. Boxes represent medians with 25th and 75th percentiles for the group. Key to animal names is presented in Fig. 3*B*. DI, Dryvax-immune (n = 8); DN, Dryvax-naive (n = 8).

suggesting both animals had been infected (Fig. 4*A*, 4*B*). 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. 4*C*).

At 2 wk postchallenge, plasma viral RNA in the control animals ranged from 6×10^6 to 2.6×10^8 copies per milliliter with median copies of 2.0×10^7/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. 3*A*, 3*B*). The median levels of plasma viral RNA in Dryvax-immune animals were 15 times lower than those in the Dryvax-naive animals (Fig. 3*A*, 3*B*). 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 control animals (p = 0.02) (Fig. 3*A*, 3*B*). At this time, the median levels of plasma viral RNA were not significantly lower in the Dryvax-naive animals than those in the control animals (Fig. 3*A*, 3*B*).

We further analyzed the viral control based on the Mamu A*01 status of the animals (Supplemental Fig. 4). The viral control in the Dryvax-immune animals was not restricted to Mamu A*01$^+$ animals, whereas in the Dryvax-naive animals it was better in Mamu A*01$^+$ animals than that in Mamu A*01$^-$ animals (Supplemental Fig. 4). In the Mamu A*01$^+$ animals, the viremia at week 2 postinfection was 20 times lower in the Dryvax-naive animals (p = 0.01) and 54 times lower in the Dryvax-immune animals (p = 0.02) than that in the unvaccinated control animals (Supplemental Fig. 4*A*, 4*B*). In the non-Mamu A*01 animals, the viremia at week 2 post infection was not significantly lower in the vaccinated animals than that in the unvaccinated animals. However, at 24 wk postinfection, it was 21 times lower in the Dryvax-immune animals (p = 0.04) but not in the Dryvax-naive animals than that in the unvaccinated controls (Supplemental Fig. 4*C*, 4*D*). 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. 3*C*, 3*D*). In the majority of the Dryvax-immune animals, the nadir of CD4 T cells did not occur until 6 wk postchallenge, whereas in the majority of animals in the other two groups, the nadir of CD4 T cells had occurred by 3 wk postchallenge (Fig. 3*C*, 3*D*). Furthermore, in four of the eight Dryvax-immune animals, the 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. 5*A*). The preservation of central memory T cells is a predictor for better survival in SIV-infected macaques (51).

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. 3*A*, 3*B*). In

Table I. *Magnitude of SIV Gag-Pol–specific IgA in rectal secretions*

Macaque	Nanograms of SIV Gag-Pol–Specific IgA per Microgram of Total IgA[a]	
	6 Wk prior to Challenge	2 Wk after Challenge
Dryvax-naive		
ROW7	0.166	0.211
RLK7	0.048	**0.226**
REP7	0.084	0.050
RNI7	**1.257**	**0.694**
RHS7	0.079	**0.733**
RGD8	0.069	0.103
RIN8	0.134	0.198
RTM7	0.081	**0.756**
Dryvax-immune		
RGR8	0.029	0.074
RVP8	0.120	0.119
RBS8	**0.372**	**0.511**
RLR8	**0.501**	0.052
RLS8	**0.948**	**1.629**
ROB8	0.064	0.198
RNK8	0.041	**0.290**
RCK8	**0.376**	**1.199**
Controls		
RPW9	0.058	0.045
RAC10	0.090	0.069
RQJ9	0.054	0.037
RRK10	0.062	0.057
RBY9	0.040	**0.338**
RYD9	0.015	0.024
RNV9	0.031	0.085
RFP9	0.032	0.178

[a]Positive responses (>0.224) are highlighted in bold.

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. 3*B*). 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. 3*E*).

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. 3*F*). All animals in which viremia peaked at week 2 had levels of colorectal virus above 10^4 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. 3*F*). 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. 3*G*). In addition, an inverse correlation was observed between the levels of colorectal virus and the frequencies of total colorectal CD4 T cells at weeks 3 ($p = 0.02$) and 24 ($p = 0.05$) postchallenge (Supplemental Fig. 5*B*, 5*C*). These results demonstrate that the enhanced control of viremia correlated with lower levels of virus at the site of challenge early postinfection.

No clear protective association between SIV-specific CD8 T cells and colorectal virus early after infection

The frequencies of virus-specific CD8 T cells in colorectal tissue and blood at 2 wk postchallenge did not correlate with reduced levels of colorectal virus (Fig. 5). To understand the role of SIV-specific CD8 T cells in viral control, we measured the SIV Gag- and Env-specific IFN-γ–producing CD8 T cells in 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. 5*A*) and blood (Fig. 5*C*) 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. 5*B*, 5*D*). Analyses for SIV-specific IFN-γ–producing CD8 T cells in blood revealed similar patterns (Fig. 5*E*, 5*F*). 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. 3*B*), the frequencies of SIV-specific CD8 T cells postchallenge showed the most variability in this group (Fig. 5*A*, 5*C*, 5*E*). 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. 5*E*).

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. 6*A*). 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. 6*B*, 6*C*). 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. 6*D*). 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. 6*E*) ($p = 0.03$). A direct correlation was also observed between the frequency of Gag-specific proliferating CD4 T cells at 4

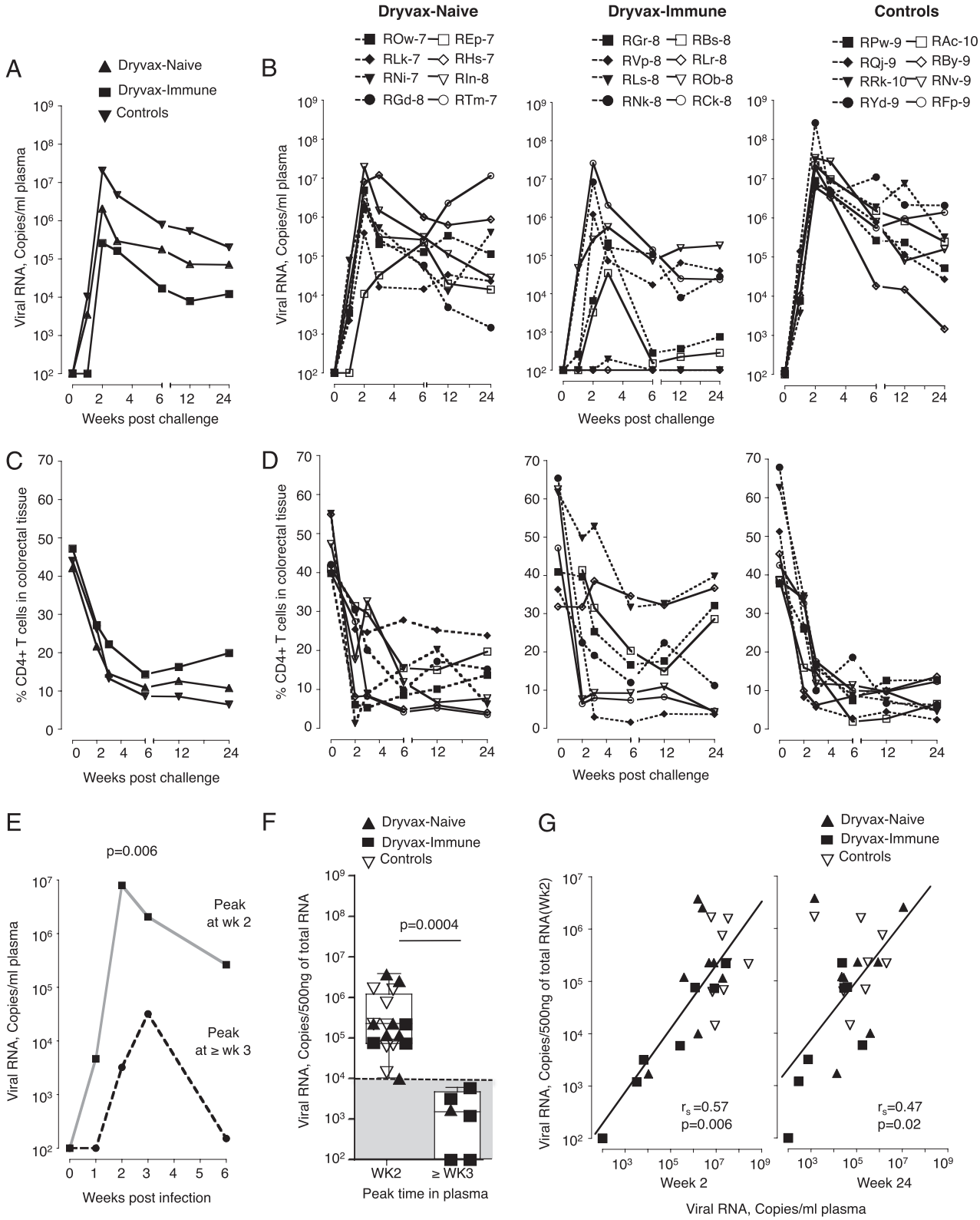

FIGURE 3. Preexisting VV immunity does not diminish the control of a pathogenic intrarectal SIV challenge. *A* and *B*, Temporal viremia median (*A*) for the group and (*B*) for individual animals. *C* and *D*, Frequency of CD4 T cells expressed as a percentage of total T cells in the colorectal tissue 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. 3*B*. 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.

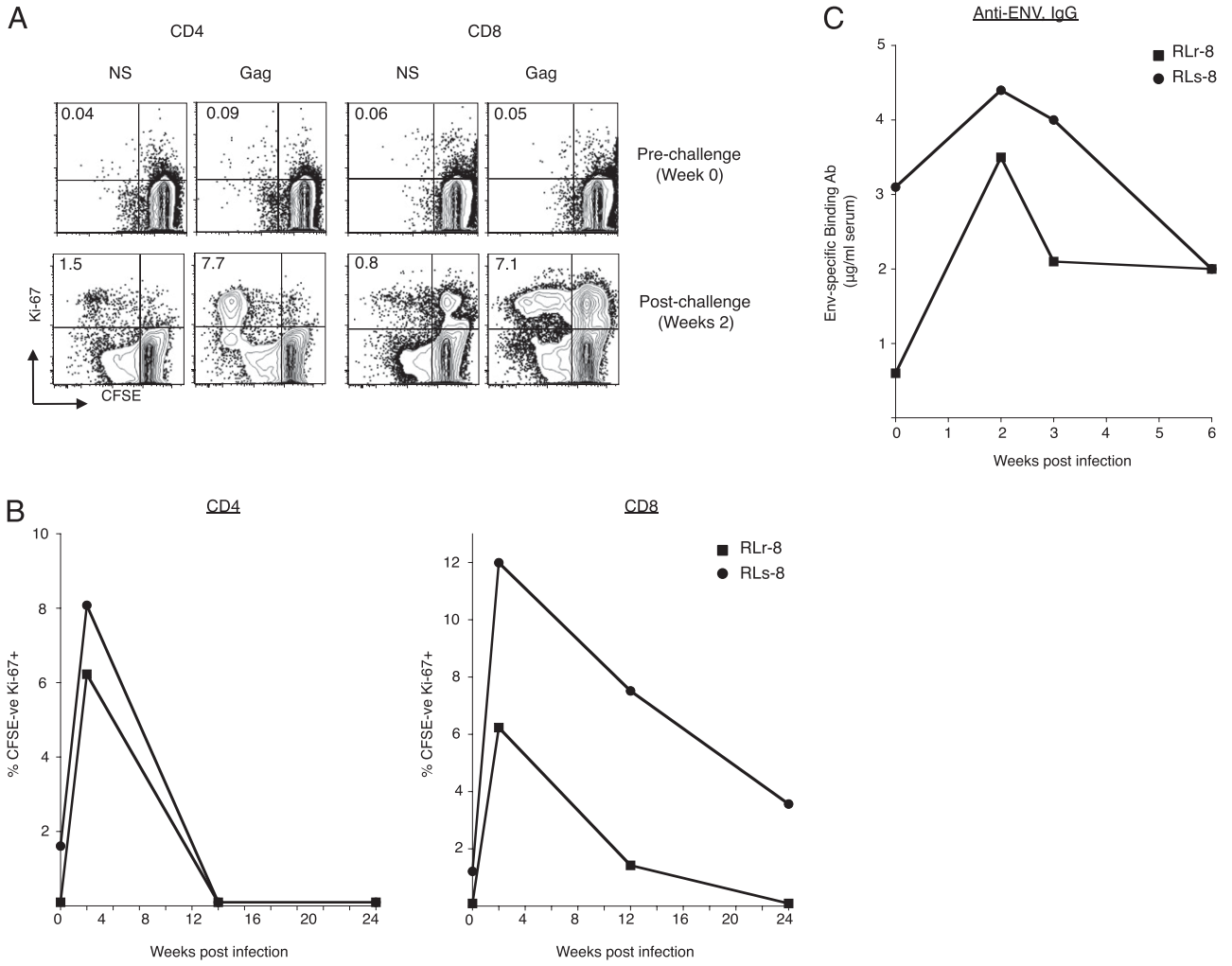

FIGURE 4. Evidence for "take" of infection in RLr8 and RLs8. *A*, Magnitude of in vitro proliferating ($Ki67^+$, $CFSE^{lo}$) SIV-specific CD4 and CD8 T cells after SIV infection. *A*, Representative FACS plots are shown demonstrating the frequency of Gag-specific CD4 or CD8 T cells after in vitro stimulation with an SIV Gag peptide pool for 6 d. Cells were gated on either $CD3^+CD4^+$ or $CD3^+CD8^+$ T cells, respectively. Numbers on the graphs represent the frequency of proliferating $CFSE^{lo}Ki67^+$ T cells as a percentage of total CD4 or $CD8^+$ T cells. *B*, Magnitude of proliferating SIV Gag-specific T cells from multiple time points are plotted for CD4 T cells on the left and CD8 T cells on the right. *C*, Magnitude of SIV Env-specific binding Ab.

wk prior to challenge and colorectal virus at 2 wk after challenge (Fig. 6*E*) ($p = 0.04$). 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 than those in the Dryvax-immune animals (Fig. 6*F*) 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-

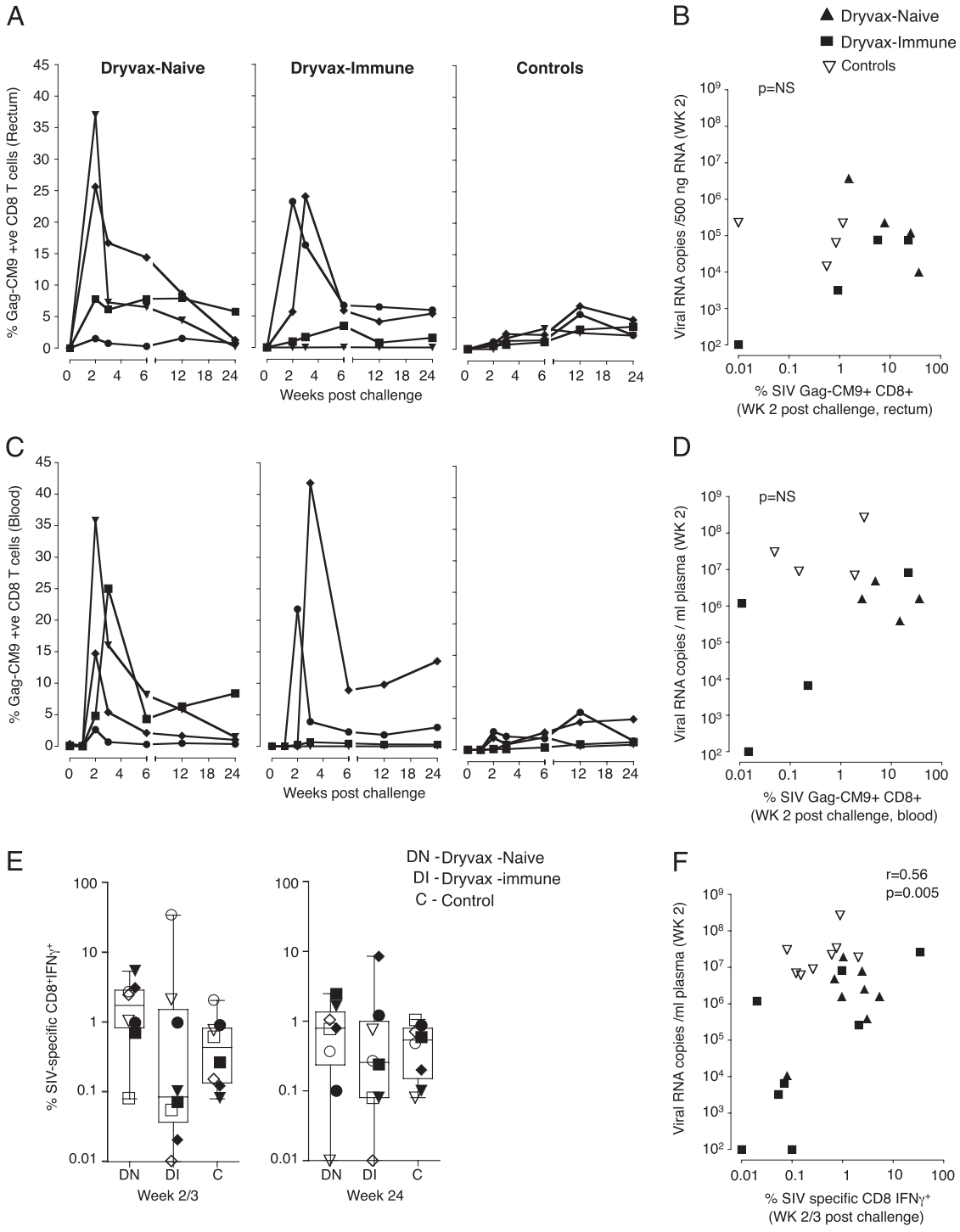

FIGURE 5. No clear association between the SIV-specific CD8 T cell response and viremia in colorectal tissue early after infection. *A*, Temporal SIV Gag-CM9–specific CD8 T cell responses in the colorectal tissue of Mamu A*01$^+$ animals. *B*, Correlation between tetramer-specific CD8 T cells and levels 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. Boxes represent medians with 25th and 75th percentiles for the group. *F*, Correlation between SIV-specific CD8 T cells and levels of virus in blood. Key to animal names is presented in Fig. 3*B*. C, control; DI, Dryvax-immune; DN, Dryvax-naive.

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

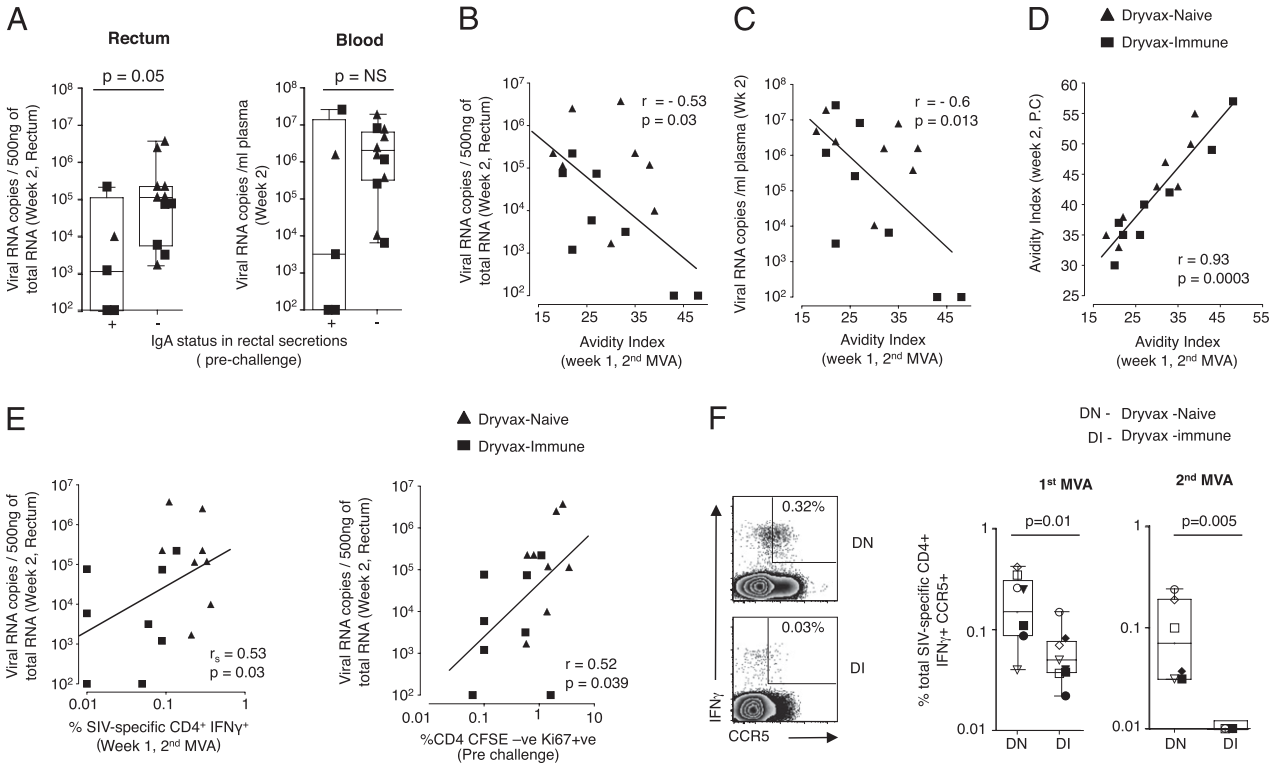

FIGURE 6. Non-CD8 T cell-mediated correlates for enhanced colorectal viral control. *A*, Comparison of levels of virus in the colorectal tissue and blood based on IgA positivity in rectal secretions prior to challenge. *B* and *C*, Correlation between avidity index after vaccination and viral load (*B*) 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. 3*B*. DI, Dryvax-immune; DN, Dryvax-naive; r_s, Spearman's rank correlation.

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 for the native form of the SIV Env correlated inversely with the levels of viral RNA in colorectal tissue at 2 wk post-SIV challenge. In contrast with the avidity indices, the titers of anti-Env binding Ab did not correlate with viral control (data not shown). These results suggest that high-avidity antiviral IgG as well as antiviral IgA contribute to colorectal viral control and highlight the importance of immunodeficiency virus vaccines eliciting these responses. We are yet to study the mechanisms by which high-avidity binding Ab and rectal IgA contribute to viral control. However, a recent study demonstrated that higher-avidity binding Ab correlates with Ab-dependent cell-mediated cytotoxicity and Ab-dependent cell-mediated virus inhibition 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 colorectal tissue at the time of and early after challenge. However, a recent study demonstrated the rapid expression of MIP-1β (ligand for CCR5) and massive recruitment of CD4 T cells to the site of infection as early as day 4 after a mucosal challenge (61) suggesting that $CCR5^+$ SIV-specific CD4 T cells from blood and other tissues may be recruited to the mucosal site of infection soon after challenge.

Two of the Dryvax-immune animals showed minimal to undetectable levels of virus infection. We think that this protection was due to vaccine-elicited immunity. Prior to challenge, these animals were the two animals in the Dryvax-immune group with the highest specific activities of antiviral IgA and the highest avidities of anti-

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.

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Disclosures

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

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