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Multiple Vaccine-Elicited Nonneutralizing Antienvelope Antibody Activities Contribute to Protective Efficacy by Reducing both Acute and Chronic Viremia following Simian/Human Immunodeficiency Virus $SHIV_{89.6P}$ Challenge in Rhesus Macaques[▽]

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We have shown that following priming with replicating adenovirus type 5 host range mutant (Ad5hr)-human immunodeficiency virus (HIV)/simian immunodeficiency virus (SIV) recombinants, boosting with gp140 envelope protein enhances acute-phase protection against intravenous simian/human immunodeficiency virus $(SHIV)_{89.6P}$ challenge compared to results with priming and no boosting or boosting with an HIV polypeptide representing the CD4 binding site of gp120. We retrospectively analyzed antibodies in sera and rectal secretions from these same macaques, investigating the hypothesis that vaccine-elicited nonneutralizing antibodies contributed to the better protection. Compared to other immunized groups or controls, the gp140-boosted group exhibited significantly greater antibody activities mediating antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cell-mediated viral inhibition (ADCVI) in sera and transcytosis inhibition in rectal secretions. ADCC and ADCVI activities were directly correlated with antibody avidity, suggesting the importance of antibody maturation for functionality. Both ADCVI and percent ADCC killing prechallenge were significantly correlated with reduced acute viremia. The latter, as well as postchallenge ADCVI and ADCC, was also significantly correlated with reduced chronic viremia. We have previously demonstrated induction by the prime/boost regimen of mucosal antibodies that inhibit transcytosis of SIV across an intact epithelial cell layer. Here, antibody in rectal secretions was significantly correlated with transcytosis inhibition. Importantly, the transcytosis specific activity (percent inhibition/total secretory IgA and IgG) was strongly correlated with reduced chronic viremia, suggesting that mucosal antibody may help control cell-to-cell viral spread during the course of infection. Overall, the replicating Ad5hr-HIV/SIV priming/gp140 protein boosting approach elicited strong systemic and mucosal antibodies with multiple functional activities associated with control of both acute and chronic viremia.

A major goal of human immunodeficiency virus (HIV) vaccine development is the elicitation of protective antibodies capable of neutralizing the diversity of isolates in the worldwide pandemic (6, 61). Indeed, passively administered neutralizing antibodies have been shown to protect against pathogenic HIV/simian immunodeficiency virus (SIV) challenge in rhesus macaque models (4, 44, 45, 57). However, the extent to which other antibody-mediated protective mechanisms impact HIV/SIV infection is still unclear. Whether these alternate biologic activities would augment vaccine-induced protection has not been definitively established.

In HIV-infected individuals, as in SIV- or simian/human immunodeficiency virus (SHIV)-infected rhesus macaques, systemic nonneutralizing antibodies appear early during acute infection, often preceding a neutralizing antibody response (21, 55). Although neutralizing antibody activity is critical for sterilizing immunity, recent studies suggest that antibodies may contribute to protection by other functional activities, such as antibody-dependent cellular cytotoxicity (ADCC) (20, 29), antibody-dependent cell-mediated viral inhibition (ADCVI) (22, 23), and transcytosis inhibition (19, 35, 59). Antibodies in secretions may directly block viral entry into intestinal and endocervical tissues by inhibiting transcytosis across epithelium, whereas local or serum-derived antibodies that mediate ADCC or ADCVI may exert protective effects by eliminating small foci of infected cells during the brief window of time that exists between transmission of virus across an epithelial cell barrier to the lamina propria and subsequent systemic spread (32). In support of this notion, mutation of the Fc portion of the broadly neutralizing monoclonal antibody, IgGb12, thereby preventing interaction with the FcγR on effector cells, rendered the antibody less able to mediate protection upon subsequent passive transfer and challenge of rhesus macaques (34). Thus, neutralizing antibodies themselves may mediate protection by additional functional activities.

ADCC bridges innate and adaptive immunity. Mechanistically, it involves FcγR-bearing effector cells, such as NK cells,

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macrophages, neutrophils, and γδ T cells, and antibodies specific for antigens expressed on the surface of target cells. Upon interaction of these three components, the target cells are killed. Since the effector cells are not major histocompatibility complex restricted, ADCC is broadly applicable to diverse populations. Because the antibody specificity need not be restricted to neutralizing epitopes, ADCC may increase the breadth of antibody reactivity. In fact, we have shown that an HIV clade B immunization regimen elicited antibodies that mediated ADCC across several HIV clades (28). Antibodies that mediate ADCC have been shown to arise early in infection, before neutralizing antibodies (55, 60). They are present in the majority of infected individuals, and they have been associated with slow disease progression following both HIV and SIV infection (5, 8).

ADCVI is closely related to ADCC, also requiring antibody that forms a bridge between an infected target cell and an FcγR-bearing effector cell (24). However, ADCVI is a broader activity not restricted solely to target cell lysis but, rather, encompassing several mechanisms by which viral replication following infection of target cells is inhibited. Thus, it may include ADCC activity but also involve noncytotoxic mechanisms of virus control, such as the secretion of inhibitory chemokines or FcγR-mediated phagocytosis of immune complexes (24, 25).

Most HIV infections occur via a mucosal route, including cervicovaginal and rectal tissues (39, 52). Several nonmutually exclusive mechanisms for HIV-1 transmission across mucosal epithelia have been proposed (13, 56). Transcytosis of infectious virus across polarized columnar epithelial cells following contact of virally infected cells with apical epithelial cell surfaces is one mechanism for mucosal HIV entry (12). Rather than fusion and infection, interactions between the viral envelope proteins and epithelial surface molecules, such as glycosphingolipid galactosylceramide (GalCer) (13, 47), an important component of endocytotic "raft" membrane microdomains, lead to transcytosis of the virus across the epithelial barrier and its trapping by submucosal dendritic cells which disseminate it to their target $CD4^+$ T cells. Studies have shown that mucosal immunoglobulin A (IgA) antibody, a major component of the mucosal immune response, could block mucosal HIV-1 entry via transcytosis *in vitro* (2, 19). Therefore, mucosal antibodies blocking adherence of virus to epithelial cells and preventing HIV-1 transcytosis across the epithelial barrier and subsequent $CD4^+$ T cell infection may afford additional protection against HIV/SIV infection.

We have been pursuing a replicating adenovirus (Ad)-HIV/SIV prime/protein subunit boost AIDS vaccine approach (30, 51), which has elicited strong, durable protection against HIV, SIV, and SHIV challenges (11, 18, 41, 42, 50). An underlying goal of these studies has been elucidation of immune responses that correlate with protective efficacy. Recently, we studied the contribution of novel protein boosts to immunogenicity and protective efficacy in a $SHIV_{89.6P}$ model (49). Immunized rhesus macaques were primed with Ad type 5 host range mutant (Ad5hr)-$HIV_{89.6P}$*gp140*, -SIV_{239}*gag*, and -SIV_{239}*nef* recombinants. One group was not boosted, one was boosted with $HIV_{89.6P}$ gp140ΔCFI protein (gp140 envelope with deletions in the cleavage site, fusion peptide, and part of the interspace between the two heptad repeats) (40), and one was boosted with a novel HIV-1 polypeptide "peptomer" representing the CD4 binding site of the envelope (54). The best protection was seen in the gp140-boosted group, with significant reductions in both acute and chronic viremia. Although Env-specific antibody and cellular responses were readily detected, none directly correlated with the better protection. Furthermore, neutralizing antibodies against $SHIV_{89.6P}$ did not develop until 4 weeks postchallenge. Therefore, we hypothesized that vaccine-elicited nonneutralizing anti-Env antibodies might have contributed to the better control of acute and/or chronic viremia in the gp140 group. Here, we report retrospective evaluations of sera and rectal secretions from macaques in this comparative study for serum binding antibody avidity, an important characteristic of functional antibodies (38, 58), and nonneutralizing activities of systemic and mucosal antibodies, including ADCC, ADCVI, and transcytosis inhibition.

MATERIALS AND METHODS

Vaccines, immunization, and challenge. The vaccines and immunization regimen were previously described in detail (49) and are summarized in Fig. 1A. Briefly, 24 Mamu-A*01-negative male Indian rhesus macaques (*Macaca mulatta*) were housed and handled at Bioqual Inc. (Rockville, MD), in accordance with the standards of the American Association for Accreditation of Laboratory Animal Care. Six macaques were included in each study group and were primed at weeks 0 (intranasally and orally) and 12 (intratracheally) with three replication-competent Ad5hr recombinants separately encoding $HIV_{89.6P}$*gp140*ΔCFI, SIV_{239}*gag*, and SIV_{239}*nef*Δ_{1-14}. Groups differed by boosting immunogens administered intramuscularly at weeks 24 and 36, receiving no boost, soluble $HIV_{89.6P}$ *gp140*ΔCFI protein, or an $HIV_{89.6P}$ "peptomer," a polypeptide representing the CD4 binding site of HIV gp120. Control macaques received empty Ad5hrΔE3 vector and phosphate-buffered saline (PBS) or monophosphoryl lipid A-stable emulsion (MPL-SE) alone. All macaques were challenged intravenously at week 44 with 90 50% monkey infectious doses (MID_{50}) of a $SHIV_{89.6P}$ challenge stock.

Sample collection. Serum samples and rectal secretions were obtained from aliquots from a previous study that were stored at −70°C. Before use in functional assays, serum samples were thawed at room temperature, diluted 10-fold with R-10 medium (RPMI 1640 containing 10% fetal calf serum [FCS], 2 mM L-glutamine, and antibiotics), and heat inactivated at 56°C for 30 min. Rectal samples were collected using cotton-tipped swabs and placed in 1 ml of PBS containing 0.1% bovine serum albumin, 0.01% thimerosal, and 750 Kallikrein inhibitor units of aprotinin. After being filtered through 0.45-μm SPIN-X tubes (Corning Inc.), they were tested for blood contamination using Chemstrips 4 (Boehringer-Mannheim). Any rectal sample that contained blood was not analyzed.

Systemic binding antibody. Serum binding antibodies to $SHIV_{89.6P}$ gp140 Env protein were assessed by enzyme-linked immunosorbent assay (ELISA) as described previously (15). Antibody titer was defined as the reciprocal of the serum dilution at which the optical density (OD) of the test serum was two times greater than that of the negative-control serum diluted 1:50.

ADCC. The rapid fluorometric ADCC (RF-ADCC) assay was performed as previously described (27). Briefly, CEM-NK^R cells (AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, Bethesda, MD) coated with $HIV_{89.6P}$ gp140 protein (ABL) were used as targets. The target cells were double stained with a membrane dye, PKH-26 (Sigma-Aldrich), and a viability dye, CFSE (Molecular Probes), before the addition of serially diluted macaque serum and human effector cells at an effector-to-target cell (E:T) ratio of 50:1. Duplicate assays were performed. Controls included unstained and single-stained target cells. Fifty thousand nongated events were acquired within 24 h of the ADCC assay using a FACSCalibur flow cytometer (BD Biosciences) and CellQuest software, setting fluorescence 1 (FL1) as the CFSE emission channel and fluorescence 2 (FL2) as the PKH-26 emission channel. Data were analyzed using WINMDI 2.9 software. The percent ADCC cell killing was determined by back-gating on the PKH-26^{high} population of target cells that lost the CFSE viability dye. ADCC titers are defined as the reciprocal dilution at which the percent ADCC killing was greater than the mean percent killing of the negative controls plus three standard deviations.

ADCVI. The ADCVI assay was based on a previously described method (23). Rhesus target peripheral blood mononuclear cells (PBMC) were stimulated with 2 μg/ml of phytohemagglutinin (PHA) (Sigma-Aldrich) and 0.5 ng/ml of recom-

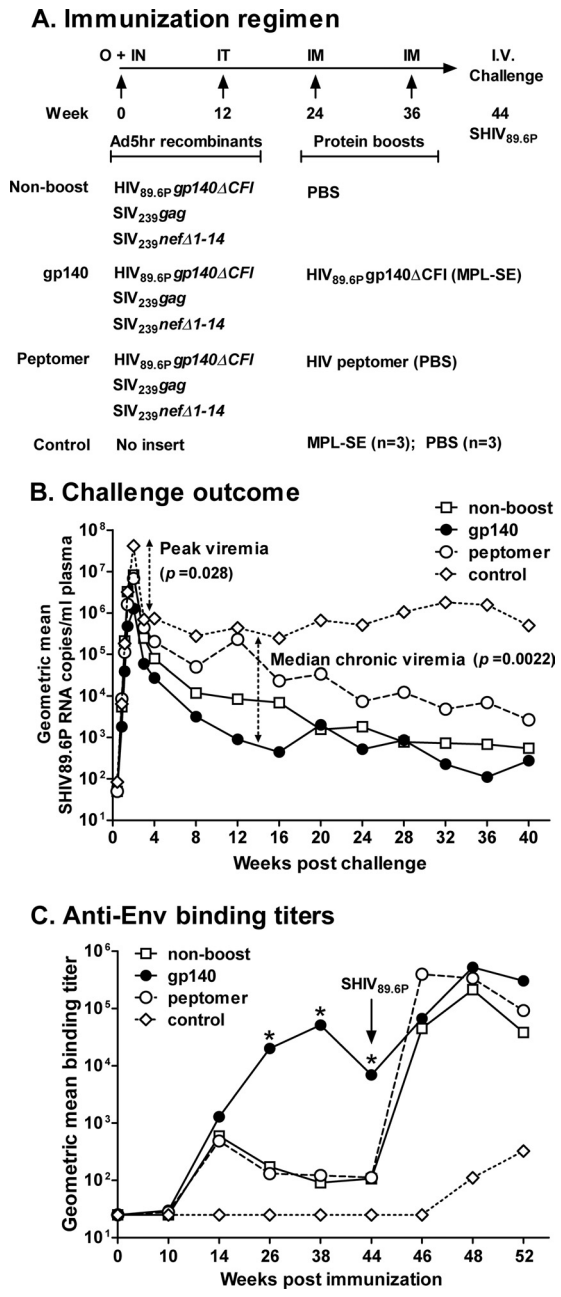

FIG. 1. Immunization regimen, challenge outcome, and anti-envelope binding titers (adapted from reference 49). (A) Three Ad5hr recombinants containing $HIV_{89.6P}$*gp140*ΔCFI, SIV_{239}*gag*, and SIV_{239}*nef*Δ_{1-14} were administered at 5×10^8 PFU/recombinant/dose, a total of 1.5×10^9 PFU/macaque either orally and intranasally (week 0) or intratracheally (week 12). Boosting immunogens were administered intramuscularly at weeks 24 and 36 at a dose of 100 μg/macaque, in either MPL-SE adjuvant or PBS as shown. All macaques were challenged intravenously at week 44 with 90 MID_{50} of a $SHIV_{89.6P}$ stock. (B) Summary of viral loads following $SHIV_{89.6P}$ challenge. Significant differences were seen in peak acute viremia and in median chronic viremia (over weeks 8 to 40) between the gp140 group and controls. (C) Anti-Env binding titers against gp140 protein. Arrow indicates time of $SHIV_{89.6P}$ challenge. The asterisks indicate statistically significant differences between the gp140 group and the nonboost and peptomer groups. The *P* values for weeks 26 and 38 ($P < 0.0002$ for both) were previously reported (49). The *P* value for week 44 is 0.0001.

binant interleukin-2 (Invitrogen) for 72 h, washed, and infected with primary $SHIV_{89.6P}$ (200 50% tissue culture infective doses). After adsorption for 1 h, cells were washed and incubated in R-10 medium at 37°C in 5% CO_2 for 48 h. Infected target cells (5×10^4/50 μl/well) were plated in 96-well round-bottom microtiter plates, and a 1/200 dilution of test serum (100 μl) was added to the target cells, along with 50 μl of rhesus effector PBMC at an effector-to-target cell ratio of 20:1. Serum in the absence of effector cells was also tested. Target cells without serum and effector cells were used as the reference control. After 7 days incubation, supernatant fluids were collected and assayed for p27 by antigen capture ELISA (ABL). The p27 levels in control wells lacking serum and effector cells ranged between 2 and 3 ng. Virus inhibition was calculated as the percentage of the decrease in p27 concentration between samples without and with effector cells, compared to the p27 concentration in the absence of both serum and effector cells, using the following formula: percent ADCVI inhibition = $100 \times \{([p27_{E-}] - [p27_{E+}])/[p27c]\}$, where [p27c] is the p27 concentration of the reference control and $[p27_{E+}]$ and $[p27_{E-}]$ are the p27 concentrations in the presence or absence of effector cells, respectively. Preimmune sera served as negative controls. A positive-control serum was not used.

Serum binding antibody avidity. The avidity of Env-specific antibody was evaluated by parallel ELISA as previously described (63). Briefly, serum samples were serially diluted and applied in duplicate to a 96-well plate coated with 1 μg/ml $SHIV_{89.6P}$ gp140 protein (ABL). After 2 h of incubation, the plate was washed and half the samples were treated with 100 μl of PBS while the paired samples were treated with 1.5 M sodium thiocyanate (NaSCN; Sigma-Aldrich) for 10 min at room temperature. Horseradish peroxidase (HRP)-conjugated goat anti-monkey IgG (AlphaDiagnostic) and the substrate, 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma-Aldrich), were used in sequential steps, followed by reading the OD at 450 nm. The avidity index was calculated by taking the ratio of the NaSCN-treated serum dilution giving an OD of 0.5 to the PBS-treated serum dilution giving an OD of 0.5 and multiplying by 100. Preimmune sera served as negative controls. A standard serum with known avidity was included on every 96-well plate.

Rectal IgA and IgG responses. Total and virus-specific IgA and IgG antibodies in rectal secretions were measured as previously described (10). Briefly, total IgA and IgG were measured by incubating serially diluted mucosal samples and a dilution series of a standard normal rhesus macaque serum with a known concentration of IgG and IgA for 2 h at room temperature on microplates coated with 1 μg/ml of purified goat anti-monkey IgA or IgG antibody (AlphaDiagnostic). Env-specific IgA and IgG were detected by incubating serially diluted mucosal samples and a dilution series of Env-specific IgA or IgG standards in microplates coated with 2 μg/ml $SHIV_{89.6P}$ gp140 protein (ABL) overnight at 4°C. The gp140-specific IgA or IgG standards, calibrated as previously described (43), were preparations of IgG-depleted pooled serum or purified serum IgG, respectively, obtained from $SHIV_{89.6P}$-infected macaques. HRP-conjugated goat anti-monkey IgA or IgG (AlphaDiagnostic) and the TMB substrate were used in sequential steps, followed by reading the OD at 450 nm. The Env-specific IgA or IgG concentration was divided by the corresponding total IgA or IgG concentration in each rectal secretion to obtain the specific activity (nanograms of antibody/micrograms of Ig).

Inhibition of transcytosis. SHIV transcytosis across epithelial cells was performed as previously described (12) with some modifications. Briefly, the intestinal epithelial HT-29 cell line was grown as a tight, polarized monolayer on 24-Transwell polycarbonate permeable membranes (6.5-mm diameter, 0.4-μm pore size; Costar, Corning) for 7 to 10 days in RPMI 1640 containing 10% fetal calf serum. The tightness of the monolayer was monitored by transepithelial resistance (>250 Ω/cm^2) with a Millicell ohmmeter (Millipore) and confirmed by a [^{14}C]inulin leakage test. The epithelial monolayer divided the well into two separate chambers: the apical (luminal) surface and the basolateral (serosal) surface. Rhesus PBMC were infected with 150 50% tissue culture infective doses ($TCID_{50}$) $SHIV_{89.6P}$. Following 3 days of culture, infected cells (5×10^5 cells/100 μl) that had been washed three times were added to the apical chamber with or without rectal secretions (100 μl of a 1:10 dilution). Transcytosis was assessed after 4 h by measuring p27 antigen in the basal chamber by antigen capture ELISA (ABL). The p27 levels in basal medium in the absence of rectal secretion ranged between 200 and 300 pg. Inhibition of $SHIV_{89.6P}$ transcytosis was calculated by obtaining the difference between the amount of p27 recovered in the basal chamber in the presence and absence of rectal sample and expressing this value as a percentage of the amount of p27 in the absence of rectal sample. Medium alone served as a negative control. A positive-control sample was not available.

Statistical analysis. The Wilcoxon rank-sum test and Wei-Johnson method were used to analyze group differences of ADCC and ADCVI activity, antibody avidity, and transcytosis inhibition at individual time points. The Spearman rank

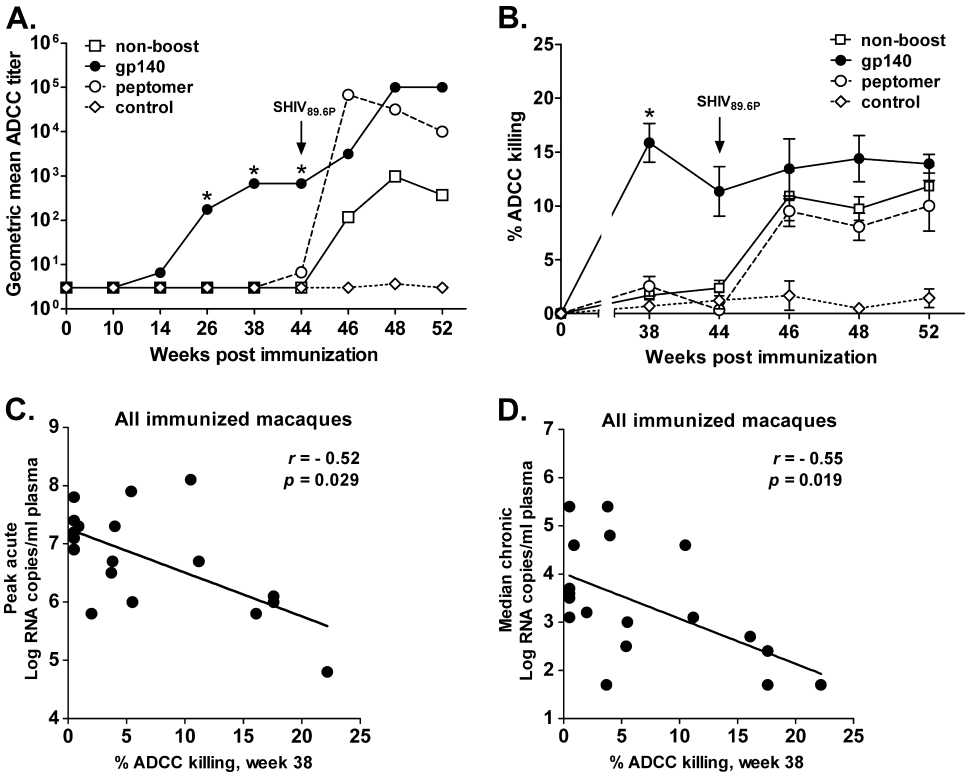

FIG. 2. Analysis of ADCC activity. (A) Geometric mean ADCC-mediating antibody titers of each immunization group. Arrow indicates time of $SHIV_{89.6P}$ challenge. *, the ADCC titer of the gp140 group was significantly higher than those of the nonboost and peptomer groups at week 26 ($P = 0.0007$) and at weeks 38 and 44 ($P < 0.0001$ for both). (B) The mean percent ADCC killing of target cells, determined at a 1:100 serum dilution and following subtraction of background killing, was significantly higher at week 38 than those of the nonboost and peptomer groups ($P = 0.0003$). Error bars indicate the standard errors of the means. Arrow indicates time of $SHIV_{89.6P}$ challenge. (C and D) The percent ADCC killing by the immunized macaques before challenge (week 38) was significantly correlated with both decreased peak acute viremia (panel C) and decreased median chronic phase viremia (panel D). The correlation coefficients (r) and P values are from Spearman rank analysis.

correlation test was used to assess the relationships between ADCC or ADCVI activity and viral load, ADCC or ADCVI activity and antibody avidity, mucosal IgA-specific activity and transcytosis inhibition, transcytosis specific activity and viral load, and serum IgG titer and rectal IgG specific activity. Because of the special status of the gp140 group observed in previous data analyses from this study (49) and because of strong correlations over time in the outcomes analyzed here, no correction of the P values for implicit or explicit multiple comparisons has been applied except where noted.

RESULTS

Env-specific ADCC in serum. As previously reported (49) and summarized in Fig. 1B, in comparison to controls the gp140-boosted group exhibited a significant reduction in both acute (day 8 to week 3) and set point (weeks 8 to 40) viremia. The nonboosted group had a similar significant reduction in set point viremia, whereas the peptomer group was not statistically different from the controls. The vaccine regimen elicited strong binding antibodies against $SHIV_{89.6P}$ envelope only in the gp140-boosted group during the course of immunization (Fig. 1C). All three immunization groups, however, exhibited anamnestic binding antibody responses postchallenge; the strongest were in the peptomer and gp140 groups (Fig. 1C). However, no statistically significant differences in binding antibody titers between immunization groups were noted postchallenge.

All immunization groups developed cellular immune responses over the course of immunization, including virus-specific memory $CD8^+$ T-cell responses, and overall the gp140-boosted group developed the most potent immunity (49). However, a direct correlation of any cellular immune response with better protection against acute- or chronic-phase viremia in the gp140 group was not observed. Furthermore, neutralizing antibodies against $SHIV_{89.6P}$ did not develop until 4 weeks postchallenge (49). Therefore, we retrospectively explored functional nonneutralizing antibody activities as a possible basis for the better protection seen in the gp140-boosted macaques. As we have previously correlated vaccine-elicited antibodies mediating ADCC with protection (20, 29), we initially investigated this functional activity in serum samples using $SHIV_{89.6P}$ gp140-coated CEM-NK^R cells as targets. Consistent with the significantly higher binding titers in the gp140 group during immunization (Fig. 1C), we also observed significantly higher ADCC titers in this group prior to challenge at weeks 26, 38, and 44 ($P = 0.0007$ for week 26 and $P < 0.0001$ for weeks 38 and 44) (Fig. 2A) than in the nonboost and peptomer groups. Postchallenge, compared with titers in the control group which never had detectable ADCC activity, ADCC titers were elevated in all three immunized groups, consistent with the strong anamnestic binding antibody responses. The gp140 group exhibited higher ADCC titers at all time points postchallenge, except at week 46, where a transiently higher ADCC titer was seen in the peptomer group.

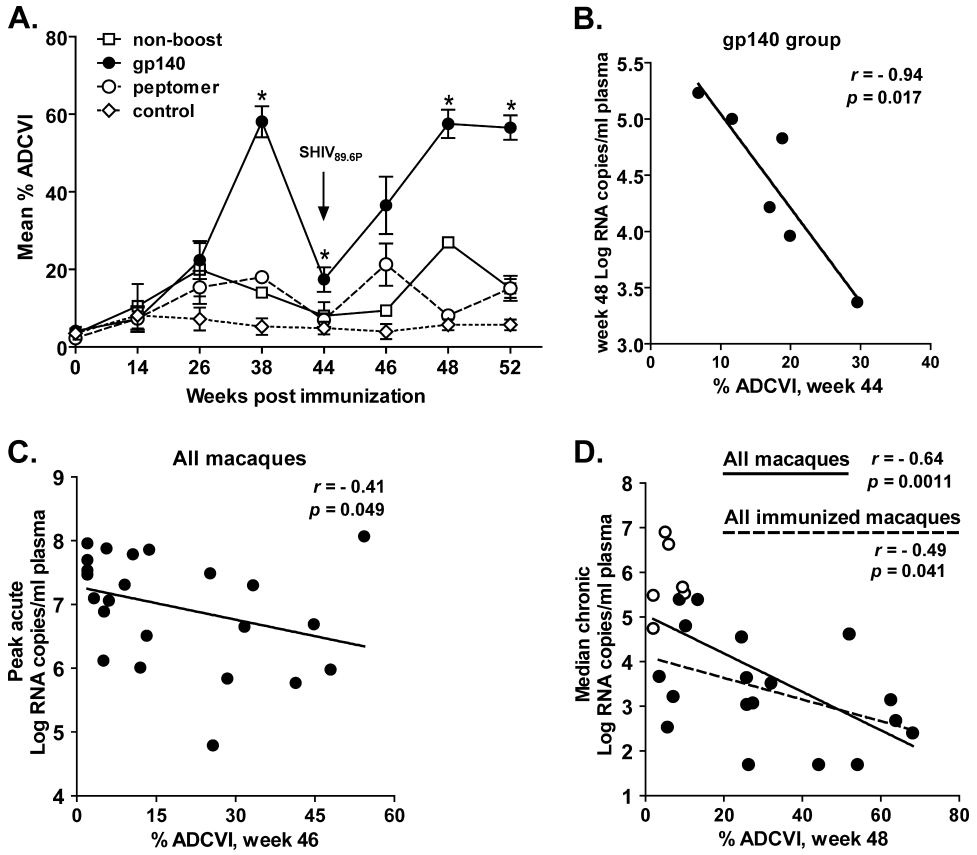

FIG. 3. Vaccine-induced ADCVI activity. (A) Mean percent ADCVI activity of each immunization group at the indicated time points. Error bars indicate the standard errors of the means. Arrow indicates time of $SHIV_{89.6P}$ challenge. *, inhibition by the gp140 group was significantly greater than that by the nonboost and peptomer groups at weeks 38 ($P = 0.0001$), 44 ($P = 0.018$), 48 ($P = 0.0001$), and 52 ($P = 0.0001$). (B) Significant inverse correlation between ADCVI activity at challenge (week 44) and week 4 postchallenge viremia in the gp140 group. (C) Inverse correlation between peak acute viremia and ADCVI activity 2 weeks postchallenge (week 46). (D) Inverse correlation between median chronic viremia and ADCVI activity 4 weeks postchallenge (week 48) in all macaques (solid line) as well as in immunized macaques only (dashed line). Open symbols represent control macaques; filled symbols represent immunized macaques. The correlation coefficients (r) and P values are from Spearman rank analysis.

Analysis of the percent cell killing by ADCC revealed that sera of the gp140 group mediated greater levels of cell killing both before and at the time of challenge than in all other groups (P value at week 38 of 0.0003) and also after challenge, although the nonboost and peptomer groups exhibited modest anamnestic responses (Fig. 2B). Moreover, the percent ADCC killing of all immunized macaques prior to challenge (week 38) was inversely correlated with both peak acute viremia ($r = -0.52$, $P = 0.029$ [Fig. 2C]) and median chronic viral loads over weeks 8 to 40 ($r = -0.55$, $P = 0.019$ [Fig. 2D]).

The correlations of postchallenge percent ADCC killing with reduced median chronic viral loads became stronger as set point viremia was approached. Taking all the macaques into consideration, correlation coefficients and P values for percent killing at the time of challenge (week 44) and at weeks 46, 48, and 52 with median chronic viremia were $r = -0.44$, $P = 0.032$; $r = -0.56$, $P = 0.0051$; $r = -0.73$, $P < 0.0001$; and $r = -0.75$, $P < 0.0001$, respectively (data not shown). This pattern reflects the increasing titers of antibodies mediating ADCC in the immunized macaques but not in the control animals.

The effect of ADCC activity on reduced chronic viremia appeared to be functioning independently of developing *de novo* neutralizing antibody. We previously reported that following challenge, the macaques in this study began to develop detectable neutralizing antibodies at week 48, 4 weeks postchallenge (49). However, the neutralizing antibody titers evaluated over weeks 48 to 64 were not significantly correlated with either ADCC titers or percent killing at weeks 48 and 52 (data not shown).

ADCVI by serum. We further evaluated another nonneutralizing functional antibody activity, ADCVI, which measures inhibition of viral replication following $SHIV_{89.6P}$ infection of target cells in the presence of FcγR-bearing effector cells and immune sera. Similarly to the Env-specific ADCC results, a consistently higher ADCVI activity was seen in the gp140 group than in other immunized groups at all time points analyzed (Fig. 3A). The ADCVI activity mediated by serum obtained at week 38 after the second gp140 boost was significantly higher in the gp140 group than in the nonboost and peptomer groups ($P = 0.0001$). All immunization groups exhibited low-level ADCVI activity at the time of challenge (week 44) although the gp140 group maintained higher levels than the other immunization groups ($P = 0.018$). The gp140 group displayed a maximum anamnestic response by 4 weeks

postchallenge (week 48), significantly higher than that of the nonboost and peptomer groups ($P = 0.0001$). The gp140 group maintained this significantly higher ADCVI activity, compared with those of the nonboost and peptomer groups, an additional 8 weeks postchallenge (week 52, $p = 0.0001$).

A strong inverse correlation was observed in the gp140 group between ADCVI activity at the time of challenge and acute viremia 4 weeks postchallenge ($r = -0.94$, $P = 0.017$ [Fig. 3B]) but not with peak acute viremia. However, in the macaques overall, ADCVI activity 2 weeks postchallenge (week 46) was significantly correlated with reduced peak acute viremia ($r = -0.41$, $P = 0.049$ [Fig. 3C]). ADCVI activity at 4 weeks postchallenge (week 48) was significantly correlated with reduced median chronic viremia in all the macaques ($r = -0.64$, $P = 0.0011$) as well as in all the immunized macaques ($r = -0.49$, $P = 0.041$) (Fig. 3D).

In contrast to the ADCC activity, ADCVI activity did exhibit a relationship with neutralizing antibody titer. While ADCVI activity at week 48 (4 weeks postchallenge) was not correlated with week 48 neutralizing antibody titer, it did exhibit a significant negative correlation with the week 52 neutralizing antibody titer ($r = -0.49$; $P = 0.039$; data not shown). This correlation became progressively weaker and ultimately nonsignificant with the week 64 neutralizing antibody titers ($r = -0.40$; $P = 0.10$). Nevertheless, evaluation of the geometric mean neutralizing antibody titer over weeks 52 to 64 revealed a significant negative correlation with week 48 ADCVI activity ($r = -0.59$; $P = 0.012$; data not shown).

Avidity of Env-specific serum antibody. Antibody avidity is an important characteristic of functional antibodies. For example, low antibody avidity has been associated with poor protective efficacy of an RSV vaccine (17) whereas high-avidity, nonneutralizing HIV-1 Env-specific antibodies have been correlated with reduced SHIV viremia following challenge (63). Therefore, we next investigated the avidity of Env-specific binding antibody in the three immunization groups. As shown in Fig. 4A, a detectable avidity index, first seen in sera of the gp140 group at week 26, was greatly increased after the second protein boost at week 38. This higher avidity was consistently seen in the gp140 group over time in comparison to modest levels in the nonboost and peptomer groups. A statistically significant difference between the gp140 group and the other two immunization groups was seen prechallenge (weeks 38 and 44) and postchallenge (weeks 46 and 48) (P values of 0.0007, 0.0019, 0.0001, and 0.0005, respectively).

Importantly, significant correlations were seen between antibody avidity and both functional antibody activities: ADCC and ADCVI. As shown in Fig. 4B, antibody avidity prior to challenge at week 38 was significantly correlated with percent ADCC killing. The correlation was slightly weaker at the time of challenge (week 44) but subsequently became progressively stronger. At weeks 44 and 46, rank correlation coefficients and P values for avidity and percent ADCC killing were $r = 0.52$, $P = 0.011$, and $r = 0.71$, $P = 0.0002$, respectively (data not shown). By week 48, the values were $r = 0.83$, $P < 0.0001$ (Fig. 4C).

Avidity was similarly correlated with ADCVI activity. Prechallenge at week 38, both the gp140 group alone as well as all macaques taken together exhibited significant correlations with ADCVI (Fig. 4D and E). As with the relationship of avidity and percent ADCC killing, the strength of the correlation between avidity and ADCVI declined at the time of challenge, week 44 ($r = 0.57$, $P = 0.0046$ [data not shown]), but became progressively stronger with time (week 46: $r = 0.64$, $P = 0.0010$ [data not shown]; week 48: $r = 0.82$, $P < 0.0001$ [Fig. 4F]). The temporal relationships between antibody avidity and both ADCC and ADCVI activities suggest that antibody maturation contributes to the functional activities.

As expected based on the significant correlations of ADCC and ADCVI with both antibody avidity and reduced viremia, avidity itself was also correlated with decreased viremia. As shown in Fig. 5A, avidity at week 46 (2 weeks postchallenge) was significantly correlated with reduced peak acute viremia ($r = -0.43$, $P = 0.037$). Moreover, avidity of all macaques as well as all immunized macaques was significantly correlated with reduced median chronic viremia ($r = -0.81$, $P < 0.0001$, and $r = -0.58$, $P = 0.013$, respectively [Fig. 5B]).

Env-specific binding antibodies in rectal secretions. An efficient HIV vaccine may need to stimulate antiviral immunity in both mucosal and systemic immune compartments, because HIV transmission occurs mostly at mucosal sites. We therefore investigated whether the immunization regimens elicited virus-specific rectal antibody responses. Due to possible variation arising from sample dilution during collections, all virus-specific antibodies were standardized to the total amount of IgA or IgG present in individual rectal samples. Induction of $SHIV_{89.6P}$ Env-specific IgA in rectal secretions was initially detected only in the gp140 group at week 22 and was enhanced by week 38 following the second protein boost (Fig. 6A). The Env-specific IgA level in rectal secretions of the gp140 group slightly decreased 2 weeks postchallenge (week 46), while anamnestic responses leading to modestly elevated levels of IgA were seen in both the nonboost and peptomer groups following $SHIV_{89.6P}$ infection. A strong anamnestic response appeared at 4 weeks postchallenge (week 48) in the gp140 group, leading to a statistically significant difference in Env-specific IgA compared to that in the nonboost and peptomer groups ($P = 0.0013$; Fig. 6A).

Env-specific IgG responses in rectal secretions exhibited a pattern similar to that of the IgA responses. Prior to challenge, they were seen primarily in the gp140 group and peaked at week 38 (Fig. 6B). All three immunization groups, however, exhibited Env-specific anamnestic antibody responses. The IgG level in rectal secretions of the peptomer group was most dramatic, rapidly increasing at 2 weeks postchallenge (week 46) compared to the nonboost and gp140 groups ($P = 0.0039$, corrected for multiple tests) and peaking at week 48. By 8 weeks postchallenge (week 52) IgG levels dropped in all immunized groups, although the values remained higher than prechallenge levels.

An examination of Env-specific rectal IgG specific activity (Fig. 6B) and serum IgG binding antibody titers (Fig. 1C) suggested that following $SHIV_{89.6P}$ exposure the IgG levels were not correlated. This would suggest that rectal IgG was produced locally postchallenge and not simply transudated from serum. Results of Spearman rank correlation analyses of these data sets are summarized in Table 1. In the immunized macaques prior to $SHIV_{89.6P}$ exposure, direct correlations between Env-specific serum and rectal IgG antibodies were ob-

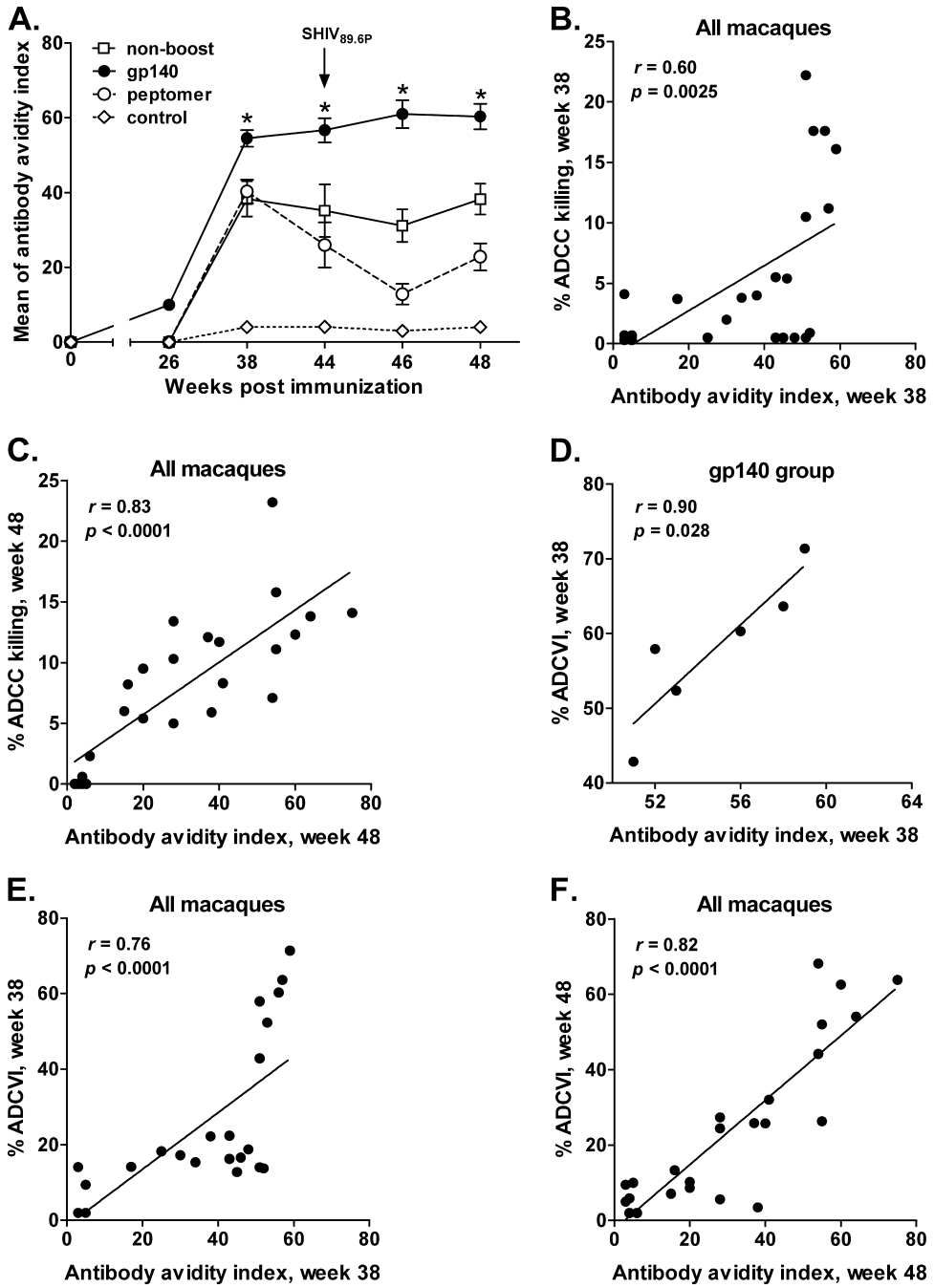

FIG. 4. Avidity of anti-Env antibody. (A) Mean of antibody avidity in each immunization group at the indicated time points. Error bars indicate the standard errors of the means. Arrow indicates time of $SHIV_{89.6P}$ challenge. *, the antibody avidity of the gp140 group was significantly higher than those of the nonboost and peptomer groups at weeks 38 ($P = 0.0007$), 44 ($P = 0.0019$), 46 ($P = 0.0001$), and 48 ($P = 0.0005$). (B and C) Significant correlation between the antibody avidity and percent ADCC killing at week 38 (B) and at week 48 (C). (D to F) Significant correlation between the antibody avidity and ADCVI activity at week 38 in the gp140 group (D), at week 38 in all macaques (E), and at week 48 in all macaques (F). The correlation coefficients (r) and P values are from Spearman rank analysis.

served at the three time points tested, as expected. The peak correlation coefficient was reached at week 38, 2 weeks after the second envelope protein immunization. Thereafter, however, the correlation coefficients declined and the P values became progressively larger. Statistical significance was maintained until week 52 postchallenge, when the correlation coefficient dropped dramatically and the P value became nonsignificant (Table 1). The correlations seen at weeks 38 and 52 in the immunized macaques are illustrated in Fig. 6C and D, respectively.

The control macaques did not develop antibodies during the course of immunization. *De novo* Env-specific serum and rectal

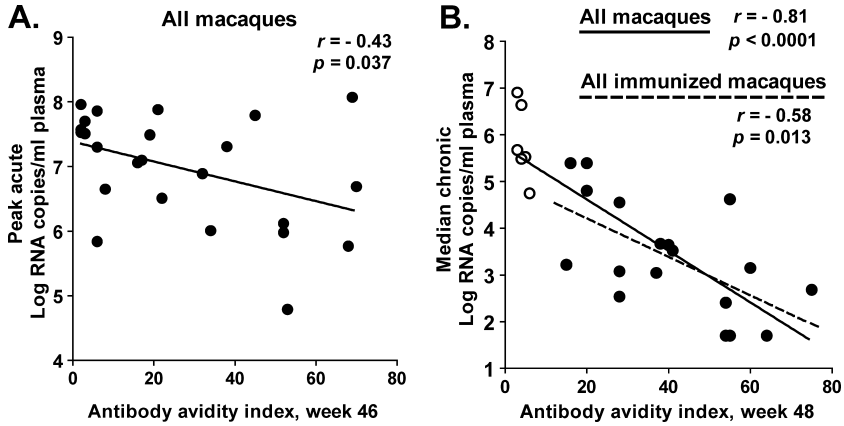

FIG. 5. Antibody avidity and viremia control. (A) Inverse correlation between antibody avidity 2 weeks postchallenge (week 46) and peak acute viremia. (B) Inverse correlation of antibody avidity 4 weeks postchallenge (week 48) and median chronic viremia in all macaques (solid line) and in immunized macaques only (dashed line). Open symbols represent control macaques; filled symbols represent immunized macaques. The correlation coefficients (r) and P values are from Spearman rank analysis.

IgG antibodies first appeared postchallenge in the controls at week 48 (Fig. 1C and 6B) and exhibited a correlation coefficient of 0.61, although the P value was not significant, perhaps due in part to the small number of macaques ($n = 6$). By week 52, the direct correlation between serum and rectal IgG antibodies in the control macaques reached 0.94, with a significant P value of 0.017 (Table 1). In this study, the SHIV challenge was administered intravenously. Nevertheless, the gastrointestinal tract is a major site of viral infection. The lack of correlation between serum and rectal IgG in the immunized macaques at week 52, in contrast to the positive correlation between these parameters in the controls, supports the notion that rectal IgG can be secreted locally following SHIV infection of vaccinated macaques rather than arising totally from transudation of serum antibody.

Transcytosis inhibition. To test the functionality of vaccine-elicited mucosal antibodies, we assayed the capacity of rectal secretions to inhibit the transcytosis of $SHIV_{89.6P}$ across a tight epithelial cell barrier *in vitro*. As shown in Fig. 7A, significantly higher transcytosis inhibition by rectal secretions from the gp140 group prechallenge (week 38) and postchallenge (week 48) than in the other immunization groups was observed ($P = 0.0047$ and 0.0001, respectively). IgA specific activity at week 48 was significantly correlated with transcytosis inhibition at the same time point in the macaques overall ($r = 0.75$, $P < 0.0001$ [data not shown]) and in the immunized macaques alone ($r = 0.67$, $P = 0.0031$ [Fig. 7B]).

Importantly, transcytosis inhibition was related to viremia control. Transcytosis specific activity (percent inhibition/total rectal IgA and IgG) prechallenge at week 38 was inversely correlated with the viral load at week 3 postchallenge in the immunized macaques ($r = -0.70$, $P = 0.0018$ [data not shown]), while an inverse correlation with the peak acute viral load in the immunized macaques was marginally nonsignificant ($r = -0.46$, $P = 0.054$ [Fig. 7C]). Strikingly, however, the transcytosis specific activity (percent inhibition/total rectal IgA and IgG) at week 48 (4 weeks postchallenge) had a strong inverse correlation with the median chronic viral load (weeks 8 to 40) in all macaques ($r = -0.63$, $P = 0.0012$ [data not shown]) and in the immunized macaques alone ($r = -0.58$, $P = 0.013$ [Fig. 7D]). Taken together, these data suggest that mucosal antibodies may help control cell-to-cell viral spread during the course of infection.

DISCUSSION

Here we have shown that a replication-competent Ad5hr-recombinant prime/envelope protein boost vaccination regimen elicits nonneutralizing antibody activities that significantly correlate with better acute- and chronic-phase viremia control following a $SHIV_{89.6P}$ viral challenge. Importantly, the envelope protein boost component of the vaccine regimen was critical in inducing high-titer antibodies and for development of rapid anamnestic antibody responses mediating ADCC killing, ADCVI, and transcytosis inhibition in the gp140-boosted group in comparison to the nonboosted, peptomer-boosted, or control macaques.

The value of the envelope protein boost in inducing nonneutralizing but functional antibody activities associated with protection is noteworthy and timely, in view of the recent outcome of the RV144 phase IIb vaccine trial in Thailand (53). It has been suggested that the modest but statistically significant protection against HIV acquisition afforded by the combined ALVAC-HIV recombinant prime/AIDSVAX (gp120) boost regimen in the RV144 study might have been due at least in part to nonneutralizing antibodies, since neither the vector nor the protein component of the vaccine has previously elicited neutralizing antibody. In fact, a previous study showed that the combined regimen can elicit ADCC activity, although the ALVAC component alone cannot (36). Further, multiple immunizations with the AIDSVAX protein component have been associated with ADCVI activity (22). Here, the contribution of an envelope boost to protection was illustrated in rhesus macaques, as two administrations of the gp140 envelope component, following Ad5hr-recombinant priming, were sufficient to elicit strong antibody activities associated with reduced viremia. In contrast to the outcome of the RV144 human trial, protection from SHIV acquisition was not achieved; however, this may reflect the single high-dose intravenous challenge used here in the nonhuman primate protocol. Others

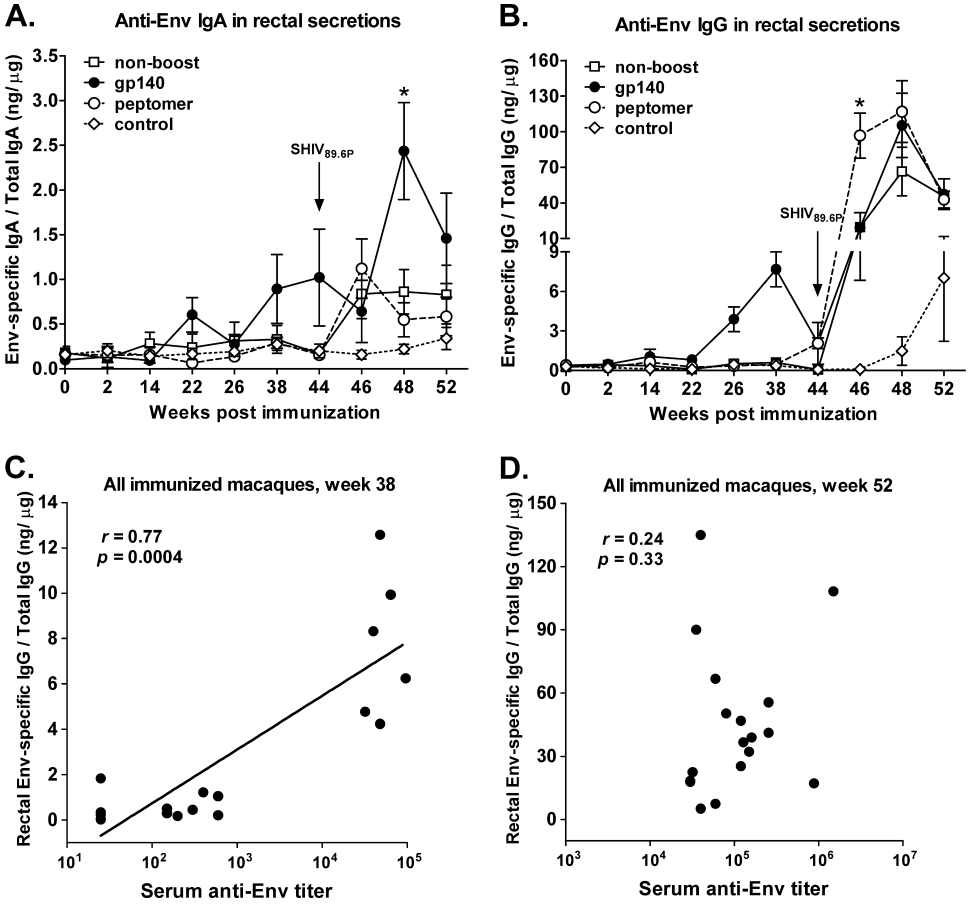

FIG. 6. Vaccine-induced mucosal antibody responses. (A) Means of Env-specific IgA in rectal secretions of each immunization group at the indicated time points. *, the level of mucosal IgA of the gp140 group was significantly higher than those of the nonboost and peptomer groups at week 48 ($P = 0.0013$). (B) Mean of Env-specific IgG in rectal secretions of each immunization group at the indicated time points. *, the level of mucosal IgG of the peptomer group was significantly higher than those of the nonboost and gp140 groups at week 46 ($P = 0.0039$). In panels A and B, error bars indicate the standard errors of the means. An arrow indicates time of $SHIV_{89.6P}$ challenge. (C and D) Correlation between Env-specific serum IgG binding titer and rectal IgG specific activity (Env-specific IgG/total IgG) at week 38 (C) and lack of correlation at week 52 (D).

have suggested that a repetitive low-dose challenge is more comparable to the human situation (37, 46, 62), and in fact protection from systemic infection following repeated low-dose challenge has been demonstrated with some immunized macaques (33). Protective efficacy elicited by our replicating Ad-recombinant prime/envelope boost regimen against a repetitive low-dose challenge will be evaluated in future preclinical studies.

In earlier vaccine studies, control of viremia following $SHIV_{89.6P}$ challenge has been attributed to vaccine-elicited cellular immune responses. Here, our demonstration that vaccine-elicited nonneutralizing antibodies can contribute to control of both acute and chronic viremia suggests an alternate interpretation of the earlier findings, with perhaps a larger role for antibody responses than previously appreciated. In several studies in which Env immunogens were incorporated, neutralizing antibodies against $SHIV_{89.6}$ and/or $SHIV_{89.6P}$ developed postchallenge in the majority of immunized macaques, but in few or none of the control animals (3, 7, 40). As the neutralizing antibodies appeared late, did not correlate with viremia control, and were not present at the time of challenge, they were not considered a protective mechanism. However, other functional antibody activities were not evaluated and, as shown here, may have contributed to the protective outcome. It would be of interest to retrospectively assay sera from these studies to uncover possible correlations of one or more antibody activities with control of viremia. Such findings would extend our observations and alter the current view that only cellular immune responses are associated with control of $SHIV_{89.6P}$ replication.

Of the nonneutralizing antibody activities mediated by serum antibodies, both ADCC killing of target cells and ADCVI activity were correlated with reduced viremia. With regard to ADCC activity, we have previously reported that this functional antibody activity elicited by vaccination was correlated with reduced acute SIV viremia (29, 35). Additionally, we have shown that vaccine-induced percent ADCC killing following a $SHIV_{89.6P}$ challenge was significantly higher and more sustained postchallenge in the best-protected group, immunized by a Tat/Env vaccine regimen, than in the group immunized by a multigenic regimen (20). Here, using a similar $SHIV_{89.6P}$ challenge we confirm and extend the earlier finding, showing

TABLE 1. Correlations of Env-specific serum IgG binding antibody with rectal Env-specific IgG, pre- and postchallenge[b]

Group and time point	Correlation coefficient (r)[a]	P value
Immunized macaques		
Prechallenge		
Week 26 (post-first protein boost)	0.55	0.019
Week 38 (post-second protein boost)	0.77	0.0004
Week 44 (time of challenge)	0.65	0.0043
Postchallenge		
Week 46 (2 weeks postchallenge)	0.60	0.010
Week 48 (4 weeks postchallenge)	0.58	0.013
Week 52 (8 weeks postchallenge)	0.24	0.33
Control macaques		
Postchallenge		
Week 46 (2 weeks postchallenge)	No antibody	
Week 48 (4 weeks postchallenge)	0.61	0.18
Week 52 (8 weeks postchallenge)	0.94	0.017

[a] Spearman rank correlation coefficient.
[b] Correlations between serum anti-Env binding titers and rectal IgG specific activity (Env-specific IgG/total IgG) were analyzed.

that the percent ADCC killing in both the immunized macaques only and all the macaques as a group was significantly correlated with reduced acute as well as chronic viremia. That ADCC activity may impact the chronic phase of infection supports earlier findings with HIV-infected people, for whom ADCC activity was associated with slower disease progression and reduced viremia (1, 8, 16, 24, 48).

The observation of a correlation between ADCVI activity and decreased acute viremia is consistent with our previous results following both SIV and SHIV challenges (20, 35). Earlier reports have associated ADCVI activity with a decreased rate of HIV acquisition (22) and protection against oral SIV infection of newborn macaques (23). Again, we did not observe any such effect here, perhaps reflecting the single high-dose intravenous challenge which may have prevented detection of a modest effect on prevention of infection.

Postchallenge ADCVI activity was associated with reduced chronic-phase viremia, in spite of a possible influence on this outcome by the relationship between ADCVI and neutralizing antibody titer. The basis for the inverse correlation between week 48 ADCVI activity and geometric mean neutralizing antibody titers over weeks 52 to 64 is not known. The ADCVI

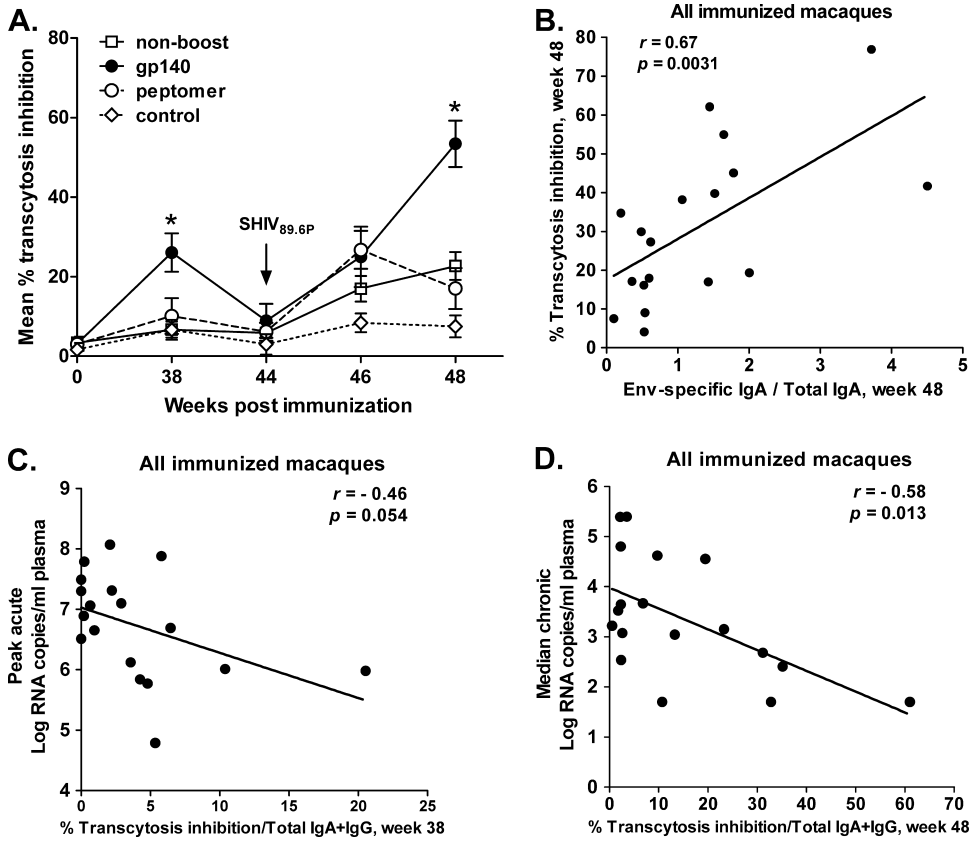

FIG. 7. Inhibition of transcytosis. (A) Mean percent transcytosis inhibition of each immunization group at the indicated time points. Error bars indicate the standard error of the mean. Arrow indicates time of $SHIV_{89.6P}$ challenge. *, inhibition by the gp140 group was significantly higher than that by the nonboost and peptomer groups at week 38 ($P = 0.0047$) and week 48 ($P = 0.0001$). (B) A significant correlation between transcytosis inhibition at week 48 and IgA specific activity (Env-specific IgA/total IgA) in the immunized macaques at week 48. (C) A marginally nonsignificant correlation between transcytosis specific activity (percent inhibition/total rectal IgA and IgG) prechallenge (week 38) and reduced peak acute viremia in the immunized macaques. (D) A significant correlation between median chronic viral loads (weeks 8 to 40) and transcytosis specific activity (percent inhibition/total rectal IgA and IgG) of the immunized macaques at week 48. The correlation coefficients (r) and P values are from Spearman rank analysis.

assay evaluates viral inhibition in the presence of serum plus effector cells and subtracts inhibition observed with serum in the absence of effector cells. The latter inhibition is attributed to neutralizing antibody. Therefore, an inverse correlation between the two activities might indicate that neutralizing as well as nonneutralizing antibodies are mediating ADCVI. Neutralizing monoclonal antibodies are known to mediate ADCVI activity (34). Alternatively, development of *de novo* neutralizing antibody depends on the presence of sufficient viral antigen to drive the antibody response. The inverse relationship between ADCVI and the more slowly developing neutralizing antibody may reflect control of viremia by ADCVI and/or other immune mechanisms at the expense of strong neutralizing antibody induction due to a reduced viral burden. In this regard, week 52 (8 weeks postchallenge) neutralizing antibody titers were significantly correlated with viral loads at the same time point ($r = 0.59$; $P = 0.012$ [data not shown]), reflecting neutralizing antibody induction in response to acute viremia. However, at the next tested time point (week 56, 12 weeks postchallenge), the correlation weakened and became nonsignificant ($r = 0.45$; $P = 0.061$). The complexity of the *in vivo* situation will make the relationships between the various functional antibody activities and viral burden difficult to resolve.

The direct correlations of antibody avidity with increased ADCC percent killing and ADCVI activity observed here suggest that antibody maturation following vaccination is associated with better functional antibody activity. This goes hand in hand with the observed associations of antibody avidity with better viremia control. Antibody avidity is a measure of the strength of the binding interaction between an antigen with multiple antigenic determinants and multivalent antibodies (58). It develops in germinal centers as a consequence of somatic hypermutation of immunoglobulin genes and selection of B cells for high-affinity binding to antigen (9, 26, 31). This antibody maturation process is therefore dependent on both time and antigen exposure. Here, all immunization groups developed antibodies with a range of avidities (Fig. 4A), perhaps reflecting the initial priming immunizations with replication-competent Ad5hr-recombinants which provided persistent antigen exposure. However, as greater avidity and functional antibody activities were seen in the gp140-immunized group, the envelope protein boost clearly contributed to enhanced antibody maturation. The basis for this may have included not only the additional antigen exposure but also the prolonged immunization regimen, in which the priming and boosting immunizations were 3 months apart. While the nonboosted and peptomer groups displayed potent anamnestic binding antibody responses following viral challenge, reflecting the priming immunizations with the Ad5hr-recombinant (Fig. 2A), both groups in comparison to the gp140 group exhibited lower avidity indices, reflecting the lack of antigen exposure in the former case and the restricted boosting by the single epitope peptomer, a polypeptide composed of repeating 18-mers representing a portion of the C4 domain of gp120, in the latter case. Greater avidity is elicited by multideterminant antigen and multivalent antibody interactions.

With regard to mucosal immunity, an important contribution of the gp140 protein immunizations was in boosting rectal IgA antibodies. Elevated levels were seen in the gp140 group both before and after challenge. Overall, the Env-specific IgA levels in the immunized macaques were directly associated with functional activity that mediated inhibition of $SHIV_{89.6P}$ transcytosis. Dimeric IgA specific for HIV envelope protein has been shown to block transcytosis of primary HIV isolates (14). The limited rectal secretions available from our macaques did not allow further purification and characterization of the mucosal IgA or direct assessment of its ability to mediate transcytosis inhibition. This demonstration will await future studies.

Here, transcytosis inhibition associated with the anamnestic response postchallenge was correlated with reduced chronic viremia over weeks 8 to 40 postchallenge. Transcytosis inhibition, and therefore blocking of viral transmission, has been suggested to be responsible for continued protection of highly HIV-exposed but persistently seronegative individuals (19). The significant correlation with reduced chronic viremia seen here suggests that mucosal IgA present in the submucosa may play a broader role in viremia control by inhibiting viral spread postchallenge. Further studies will be required to explore this hypothesis. Notably, the rectal secretions in immunized macaques contained IgG which by 8 weeks postchallenge was not correlated with serum IgG, suggesting local secretion in response to SHIV infection. The induction of such antibody and its contribution to control of chronic viremia merit further investigation. The fact that the lack of correlation was seen in immunized but not control macaques suggests that vaccine-induced memory B cells which traffic to the mucosa may play a role.

Here, we demonstrate the value envelope boosting provides to a vaccine regimen based on priming with a vector targeted to the mucosa. Not only were anti-envelope antibodies boosted to higher titers; they exhibited greater functional activity and increased avidity. Importantly, functional mucosal antibodies were also elevated. These benefits were obtained using an envelope monomer that has not elicited neutralizing antibody against the challenge virus. One would expect greater benefits when boosting is conducted with an envelope vaccine possessing the appropriate oligomeric conformation able to elicit neutralizing antibodies.

Overall, our data indicate that multiple functional activities of nonneutralizing systemic and mucosal antibodies had a significant impact on the challenge outcome, resulting in better acute- and chronic-phase protection of the macaques immunized by the replicating Ad5hr-HIV/SIV-priming/gp140 protein-boosting regimen. Significant levels of systemic and mucosal immune responses and greater protection from infection were achieved in our study, providing a strong rationale for continued development of the recombinant prime/envelope boost approach. As additional functional antibody activities associated with protection are identified, further improvement in the design of the envelope protein used for boosting in an HIV vaccine regimen will be necessary to elicit not only broadly neutralizing antibodies but also the spectrum of nonneutralizing antibody activities.

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