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Chemokine Adjuvanted Electroporated-DNA Vaccine Induces Substantial Protection from Simian Immunodeficiency Virus Vaginal Challenge

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Abstract

There have been encouraging results for the development of an effective HIV vaccine. However, many questions remain regarding the quality of immune responses and the role of mucosal antibodies. We addressed some of these issues by using a simian immunodeficiency virus (SIV) DNA vaccine adjuvanted with plasmid-expressed mucosal chemokines combined with an intravaginal SIV challenge in rhesus macaque (RhM) model. We previously reported on the ability of CCR9 and CCR10 ligand (L) adjuvants to enhance mucosal and systemic IgA and IgG in small animals. In this study, RhMs were intramuscularly immunized five times with either DNA or DNA plus chemokine adjuvant delivered by electroporation followed by challenge with SIVsmE660.

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Sixty-eight percent of all vaccinated animals (P=0.0016) remained either uninfected or had aborted infection compared to only 14% in the vaccine naïve group. The highest protection was observed in the CCR10L chemokines group, where 6 of 9 animals had aborted infection and two remained uninfected, leading to 89% protection (P=0.0003). The induction of mucosal SIV-specific antibodies and neutralization titers correlated with trends in protection. These results indicate the need to further investigate the contribution of chemokine adjuvants to modulate immune responses and the role of mucosal antibodies in SIV/HIV protection.

Although a large number of vaccines have been tested, after a 30-year effort, there is still a need for a highly efficacious HIV-1 vaccine. The recent RV144 clinical vaccine trial in Thailand demonstrated that 31% of vaccinated individuals could be protected[1-3]. The need for an effective HIV-1 vaccine to extend positively on these results remains pressing. DNA based vaccines alone have been shown to induce weak immune responses in non-human primates (NHP) and humans thus limiting their stand-alone utility. However, many technological advances to the platform have recently resulted in improving this platform's performance in the clinic[4,5]. Such advances include using codon and RNA optimization, electroporation, and the use of genetic adjuvants to tailor the immune response[6-15]. The potency of plasmid adjuvants for DNA vaccines was recently demonstrated in HVTN080 trial, reporting that the inclusion of pIL- 12 (plasmid encoded IL-12) in a DNA + EP formulation in humans increased vaccine induced responses[5]. In this study following three immunizations, 88.9% of vaccinated subjects developed $CD4^+$ or $CD8^+$ responses. However, an effective HIV vaccine will likely need to also induce antibody responses[2,16]. The role of antibodies in protection has been supported by the immune correlates analysis of RV144 and in several studies using passive transfer of broadly neutralizing antibodies to NHPs resulting in protection against challenge[1,17-23]. However, these broadly neutralizing antibodies are highly somatically hypermutated with uncommon characteristics such as long CDR3s, calling into question whether a vaccine will be able to induce such antibodies[24].

In order to increase the magnitude and quality of humoral responses induce by DNA vaccination, we explored the use of mucosal chemokine plasmid adjuvants in combination with a SIV vaccine. Previously, we determined that the CCR10L adjuvants CTACK (cutaneous T-cell attracting chemokine, or *CCL27*) and MEC (mucosa-associated epithelial chemokine, or *CCL28*) increase the levels of vaccine specific mucosal IgA and IgG in small animals[25,26]. The receptor for these two chemokines is CCR10 which is expressed on mucosal and epithelial tissue, allowing for the recirculation and localization of naïve, memory and effector T cells and antibody secreting B cells[27-34]. In addition, the chemokine TECK (thymus-expressed chemokine or *CCL25*) which binds to CCR9 has been found to be important in T cell homing to the lamina propria and intraepithelium of the small intestine[35-38]. Previous studies have also shown that the inclusion of TECK with a DNA vaccine can elevate antigen specific responses in both the serum and mucosal compartments of mice[39].

We report here that rhesus macaques (RhMs) vaccinated with SIV *gag, env,* and *pol* and CCR9L and CCR10L adjuvants delivered by electroporation can be protected from multiple low dose intravaginal challenge with SIVsmE660. When all vaccine arms were combined,

13 out of 19 animals remained uninfected or displayed aborted infection, controlling the virus to undetectable levels, leading to a total vaccine protection of 68% vs 14% in control challenged animals ($P = 0.0016$). The highest protection was seen in the DNA + CCR10L group with an 89% protection rate ($P = 0.0003$) with 6 of 9 RhMs displaying aborted infection and two RhMs remaining uninfected. The inclusion of mucosal chemokine plasmid adjuvants improved challenge outcomes by over two-fold compared to DNA alone and suggests that further study of novel immune adjuvanted vaccines are of importance.

Results

Inclusion of mucosal chemokine adjuvants induces robust cellular responses to all antigens

In this study, we vaccinated four groups of animals consisting of five female RhMs with pSIVmac239 *pol* and pSIV sooty mangabey consensus *envelope* and *gag* vaccine alone or in combination with CCR9L *pCCL25* or CCR10Ls *pCCL28* or *pCCL27* at weeks 0, 6, 12, 18 and boosted at week 48. We also vaccinated 14 female rhesus macaques with water followed by EP and termed this group "naïve" control animals (Supplemental fig 1*a*). The consensus immunogens were developed as previously described using multiple sooty mangabey SIV sequences[40-42]. The homology of the SIVsm Envelope to isolates from SIVsmE660 swarm ranges from 94-97% (Supplemental fig. 1*b*). Compared to pre-vaccination levels (Fig. 1*a*), after four immunizations, all RhMs showed robust cellular responses against all vaccine immunogens (Fig. 1*b*). RhMs immunized with CCR9L chemokine had significantly higher total amount of IFN-γ secreting PBMCs compared to DNA only ($P < 0.01$) which was predominately $CD8^+$ T cell driven (Supplemental fig. 2*a*). All animals demonstrated good recall responses after the final immunization to all included vaccine antigens (Fig. 1*c*). Cellular responses were further investigated two weeks after final immunization to determine if there were differences in cytokine profiles between the groups. Peripheral blood mononuclear cells (PBMCs) were stimulated with Gag and Pol peptides followed by intracellular cytokine staining. The inclusion of CCR9L adjuvant increased the amount of antigen specific $CD8^+$ T cells secreting IFN-γ, TNF-α and IL-2 (Fig. 1*d*). The addition of CCR10L adjuvants only marginally affected $CD8^+$ T cells, with the largest difference in TNF-α and IL-2 expression compared to the DNA only group (Fig. 1*d*). Polyfunctionality for both $CD4^+$ and $CD8^+$ T cells were also assessed after final vaccination (Fig. 1*e*), displaying limited differences in the amount of cells secreting all three cytokines but increased differences in populations secreting either two or a single cytokine.

Inclusion of mucosal chemokine adjuvants increases humoral responses in sera and secretions

Since both CCR9L and CCR10L adjuvants have previously been shown to increase humoral responses[25,26,39], we investigated the vaccine induced antibody production in serum and vaginal washes. We confirmed that the addition of CCR10L adjuvant enhanced vaccine-specific IgA above the levels induced by DNA alone vaccination. These results were obtained by measuring Gag (p27)- and Env (gp160)-specific IgA in the serum and vaginal wash by ELISA and western blot (WB) two weeks after final vaccination. The use of CCR10L adjuvant resulted significant elevation in serum IgA levels against p27 antigen

(P<0.05 compared to both DNA only and CCR9L) and against gp160 (P < 0.05 compared to DNA only) measured as WB band intensity (Fig. 2*a*). Additionally, there was a trend for increased serum IgA against gp160 observed in RhMs vaccinated with CCR9L adjuvant. However, there were no significant differences in the levels of serum SIV-specific IgA Envelope ELISA binding titers between groups (Fig. 2*e*). Serum IgG, revealed similar strong WB band intensities against p27 and gp160 in CCR10L adjuvant group (Fig. 2*b*). Moreover, endpoint titers of serum IgG antibodies to gp140 also tended to be elevated in CCR10L adjuvanted RhMs (Fig. 2*f*). Although the values are not significant, due to large variability, the p27 and gp160 IgA binding antibodies were elevated in vaginal secretions of animals receiving CCR10L adjuvants (Fig. 2*c*). Only 3 out of 5 RhMs receiving the DNA vaccine exhibited measurable IgA responses averaging 0.53 WB band intensity units. In contrast, 7 out of 9 animals receiving CCR10L adjuvants had measurable p27 IgA responses, with an average WB band intensity of 1.6. In the CCR9L adjuvant group, 4 out of 5 animals had measurable p27 IgA responses with an average WB band intensity of 1.1. Likewise, there were no detectable vaginal gp160 IgA responses in DNA-vaccinated animals, whereas 4 out of 9 animals receiving CCR10L adjuvanted vaccine had gp160-specific responses. Neither CCR9L nor CCR10L adjuvants appeared to have much of an effect on vaginal IgG responses compared to DNA only immunized group (Fig. 2*d*).

To further characterize potentially protective vaccine-induced humoral responses, we measured V1/V2 binding using a linear peptide pool ELISA and the neutralizing antibody titers using the standard TZM-bl assay. The consensus SIVsmE660 vaccine induced V1/V2 binding antibodies, but V1/V2 binding seemed to be only slightly enhanced by the addition of CCR9L or CCR10L adjuvants (Fig. 2*g*). Serum IgG binding to linear peptides against V3 and gp41 were also investigated but there was no induction of responses to these regions by the vaccine (data not show). We also observed neutralizing titers present against the tier 1 SIVsmE660.11 psuedotyped virus; however, there were no significant differences in neutralization titer between groups, with CCR9L vaccinated animals showing a slight increase compared to the other groups (Fig. 2*h*).

Inclusion of CCR9L and CCR10L adjuvants enhance protection against challenge

To assess the impact of vaccine induce responses and acquisition of SIV, we performed a repeat intravaginal challenge with 500 $TCID_{50}$ (median tissue culture infective dose) SIVsmE660 that had been previously titered for vaginal challenge. Fourteen vaccine-naïve animals were included as challenge controls. Following challenge, we observed that 12 out of 14 vaccine-naïve RhMs became infected, and all animals exhibited acute peak of viremia of 10^6 to 10^8 viral copies per ml and setpoint from 10^4 to 10^6 viral copies per ml (Fig. 3*a*). Two vaccine-naïve animals did not become infected with a baseline percent protection of 14.2. Grouping all vaccinated animals together, 13 out of 19 display either no infection or aborted infection corresponding to 68% protection (Fig. 3*b*) which is highly significant (P = 0.0016 compared to naïve). When animals were divided into their corresponding vaccine regimens, there was a large difference in challenge outcome. Two out of five DNA only vaccinated RhMs remained uninfected, leading to 40% protection (P= 0.23 compared to naïve) (Fig. 3*c*). This protection trended higher in the CCR9L vaccinated animals, in which three out of five RhMs were protected, corresponding to 60% protection (P = 0.06 compared

to naïve) (Fig. 3*d*). The challenge outcomes for animals immunized with CCR10L adjuvanted vaccine were noticeably different; 2 out of 9 RhMs remained uninfected and 1 out of 9 displayed progressive infection. The remaining 6 out of 9 animals had aborted infections, exhibiting brief viremia that rapidly declined to below detectable levels, resulting in 89% protection which is highly significant ($P = 0.003$ compared to naïve) (Fig. 3*e*).

Following challenge, we also observed significant vaccine effects on viral parameters. Compared with vaccine-naïve animals, there was a significant decrease in peak viral load in all vaccinated animals ($P<0.05$) (Fig. 4*a*), specifically in the CCR10L adjuvanted group ($P<0.05$) (Fig. 4*b*). A more dramatic adjuvant effect was observed when analyzing the time to viral control. There was a trend towards decrease time to viral control in all vaccinated compared to naïve animals (Fig. 4*c*). This was further emphasized when each group was analyzed. RhMs immunized with CCR10L adjuvants showed a significant decrease in days to viral control when compared to naïve ($P<0.001$), DNA only ($P<0.001$) and CCR9L chemokine adjuvant ($P< 0.001$) with an average time to control of 38.7 days (Fig. 4*d*). Viral loads for all uninfected and aborted infections remained below detection thru the end of the study, 6 months post challenge (Fig. 3).

Differential induction of vaginal IgA and IgG antibodies could influence outcome of vaccination

In order to further understand how differential induction of antibody isotypes could influence the challenge outcome, data analysis was performed for animals grouped according to their disease progression. Specifically, “uninfected” animals were defined as having no detectable viral loads through challenge follow up; “aborted infection” for the animals which were infected but controlled viremia to undetectable levels; and finally “progressive infection” for the animals with measurable viral loads throughout the study. Humoral responses were followed two months post challenge, a time chosen to be after the peak of viral infection. In the uninfected animals, the vaginal and serum IgA and IgG antibodies specific for envelope remained unchanged after challenge, suggesting that these animals remained truly uninfected (Fig. 5*a*-*b*). However, animals with aborted or progressive infections had significant increases in Envelope binding antibody titers in both systemic and mucosal compartments (Fig. 5*a*-*b*). In both abortive and progressive animals, vaginal antibody endpoint binding titers to Envelope increased almost 4000-fold for IgA and 30,000-fold for IgG after infection. Within the serum compartment these increases were 50,000-fold and 1,000,000-fold for IgA and IgG respectively. Additionally, serum neutralizing titers of antibodies did not change after challenge for uninfected RhMs but did increase significantly for both aborted and progressively infected RhMs (Fig. 5*c*).

To determine whether potential correlates of immunity exist for RhMs which remained uninfected or displayed aborted infection, we analyzed responses two weeks after final immunization. Due to the limited number of animals in each outcome group, the study analysis was not powered to detect small changes in antibody levels and thus there was no significant difference when evaluating individual groups. However, there were some trends of importance: including differences in the induction of vaginal IgA and IgG to viral proteins (Fig. 6*a*–*b*). Specifically, RhMs with progressive infection only exhibited vaginal

IgA and IgG antibodies to Gag (p27) whereas RhMs which remained uninfected or aborted infection displayed vaginal IgA and IgG antibodies to Envelope, Gag and Pol. For all proteins except Gag, RhMs with aborted infection exhibited the highest levels of IgA and IgG. Within the serum, all challenge outcome groups induced binding IgA and IgG antibodies to all vaccine antigens (Fig. 6*c-d*). Binding titers of serum antibodies to Envelope (gp160) did not show any difference across the groups (Fig. 6*ef*). Uninfected animals exhibited the highest level of SIVsmE660.11 neutralizing antibody titers followed by the abortively infected group (Fig. 6*g*). When investigating the V1/V2 linear epitope binding response, both uninfected and abortively infected RhMs showed higher responses than the progressively infected animals (Fig. 6*h*). The number of IFN-γ secreting T cells were similar in all outcomes indicating that peripheral T cell responses did not appear to contribute to challenge outcome (Fig. 6*i*). Total $CD4^+$ or $CD8^+$ T cells secreting cytokines after 4^{th} and 5^{th} immunization also did not appear to correlate with challenge outcome or peak viral loads (Supplemental fig 3*a-b*). Taken together, the data from this pilot study suggest that the presence of mucosal IgA and IgG and neutralization titers inversely correlate with levels of SIV infection and likely contributes to prevention of infection.

Discussion

A strength of the DNA vaccine platform is its ability to combine plasmids encoding cytokines and chemokines as part of the vaccine formulation, which are able to specifically influence the immune responses towards a desired outcome[5,8,11,14,25,26,39,43–45]. In this study, we demonstrated that the addition of immune plasmid adjuvants encoding mucosal chemokines can increase the effectiveness of a DNA vaccine against an SIV challenge. Macaques immunized with CCR10L adjuvanted vaccine demonstrated 89% protection with 6 of 9 displaying aborted infection. These animals did not exhibit positive viral loads through the end of the study, corresponding to 6 months post challenge follow up. Within the CCR10L immunized animals, only 2 out of 9 animals remained uninfected compared to 3 out of 5 for CCR9L vaccinated animals and 2 out of 5 in the DNA only immunization, suggesting different possible mechanisms of protection between the vaccinated groups. The majority of control in the CCR10L immunize animals occurs after the virus has already disseminated whereas the control in the CCR9L immunized animals blocks the establishment of infection or dissemination into the peripheral blood. Future studies investigating these differences in control could shed light on the development of an efficacy HIV-1 vaccine.

In addition to the use of a highly novel gene adjuvant, this study has many other innovative factors. These include the use of adaptive electroporation to drive increased transfection efficiency and *in vivo* expression of antigen. Within this study, we see strong protection against challenge with the use of a DNA only immunization regiment. A strength of DNA vaccination continues to be the induction of strong cellular responses but limited to no antibody responses. Due to this, we have continued to focus on increasing DNA vaccine's ability to drive systemic and compartmentalized antibody responses while trying to maintain cellular responses. Within this study, we are able to induce both strong cellular and humoral responses using only DNA without the possible serological complications of viral vectors or live attenuated vaccines. There have been few studies which have looked at the ability of

DNA vaccination to induce mucosal responses and in many cases, the addition of a heterologous boost is required[46–49]. However, within this study using only DNA, we see 15 out of 19 RhMs inducing mucosal responses as measured by WB band intensity units against either Envelope or Gag. Additionally, the constructs used within this study were not matched to the SIVsmE660 swarm and demonstrate the ability of a synthetic consensus immunogens to drive cross reactive and broad responses that can impact viral infection. The viral challenge was specifically titered for vaginal challenge, mimicking early infection from male to female while yielding a high rate of infection in naïve. Another novelty of the study is the strength of looking at both the serum and mucosal responses. The ability to induce responses in both compartments will likely be important for future HIV vaccines. Within the study we see that what is observed within the serum does not necessarily predict what is observed in the vaginal mucosa.

Though correlate analysis is difficult with smaller animal groups, we do see some trends. Compared to other platforms such as the CMV vectors, which show increase abortive infection after peak viral load[50–52], we do not observe differences in the assayed T cell responses induced between groups. Instead, all difference appeared to be related to humoral responses. As expected, uninfected RhMs have the highest titers of neutralizing antibodies to SIVsmE660.11 isolate. Subsequent analysis of RV144 trial indicated that antibodies to the V1/V2 loops of HIV envelope correlated with a lower risk of HIV infection[1,53]. Following this, RhMs which remained uninfected and abortively infected had higher level of serum IgG binding to the V1/V2 region of SIVsmE660 peptides compared to progressively infected animals. In contrast to RV144, there was no difference in serum IgA binding titers to Envelope (gp140) across all groups or a correlation between vaccine induced $CD4^+$ T cells and challenge outcome and control. These results suggest the need to further investigate the relationship between vaginal IgA and IgG antibodies in HIV protection.

Though all of the differences in immune responses detected were related to humoral responses, this does not eliminate the potential for cellular responses to play a role in protection after vaccination and additional study in this regard is warranted. We have reported in a trial with the HVTN[5], that pIL-12 can increase the number of vaccine responders in humans receiving an HIV DNA vaccine delivered by EP. A future study to compare pIL-12 alone or in combination with mucosal adjuvants in this model would be informative. Additionally, the chemokine adjuvant's effects on resident effector cells at the mucosa is also important. The presence of effector memory T cells at the initial mucosal sites of infection could allow for abortive infection to occur. Previous studies in mice have suggested that the use of the mucosal chemokine adjuvants was able to up regulate the number of cells positive for either the CCR10 or CCR9 receptor at the site of vaccination[25,26,39]. We are continuing to investigate how these cells leave the muscle and migrate to mucosal sites where they become effector cells.

Within this study we report an overall protection rate of 68% in all vaccinated RhMs against a SIVsmE660 swarm mucosal challenge vs a control rate of 14%. Within the study, there is a significant increase in protection in the CCR10L-adjuvanted animals, displaying 89%. These levels of protection from chronic progressive infection are significant and thus warrant further investigation. By including different chemokine and cytokine adjuvants including

mucosal chemokines, DNA vaccines appear to specifically focus the immune response to enhance protection. Such a mechanism is of clear clinical relevance for HIV vaccine studies.

Materials and Methods

Study Design

Groups of female rhesus macaques (*Macaca mulatta*) of Indian origin ($n = 5$ per group) were immunized at weeks 0, 6, 12, 18 and 48 with 1.5 mg per construct of pSIVmac *pol*, consensus pSIVsm *env* and 3.0 mg pSIVsm *gag* without adjuvant. Adjuvanted groups included rhCCL25 ($n = 5$), rhCCL27 ($n = 5$) or rhCCL28 ($n = 5$), at 0.5 mg. DNA was formulated in sterile water with 1% wt/wt poly-L-glutamate sodium salt and delivered in two separate sites followed by *in vivo* electroporation using the CELLECTRA® device (Inovio Pharmaceuticals, Inc.; Plymouth Meeting, PA). An additional 14 animals were treated with water followed by EP and served as a naïve control. RhMs with protective MHC allele mamu A01* were evenly distributed in order to not bias results. TRIM5α analysis was performed after challenge and did not appear to have a major impact on the overall challenge outcome (Supplemental table 1). One animal from the CCR10L immunized group died before challenge due to unrelated causes and was not included in any of the analysis.

Animal husbandry and specimen collection schedule

RhMs were housed at Tulane National Primate Research Center in accordance with the standards of the American Association for Accreditation of Laboratory Animal Care. Animals were allowed to acclimate for at least 30 days prior to any immunization. All protocols were approved by Tulane National Primate Center Animal Care and Use Committee.

Collection of whole blood from rhesus macaques

Animals were anesthetized with ketamine (0.1 ml/kg) or tiletamine/zolazepam (0.06–0.10 ml/kg). Blood samples were collected from the femoral vein using the Sarstedt S-Monovette collection system (Sarstedt; Nümbrecht, Germany). Peripheral blood mononuclear cells (PBMC) were isolated by standard Ficoll-hypaque centrifugation.

Collection of vaginal wash from rhesus macaques

After administration of anesthesia, an appropriate-sized feeding tube was carefully introduced into the vaginal vault. A syringe containing 2 ml saline was attached to the feeding tube and used to instill and aspirate the saline from the vaginal vault. The sample was transferred to a sterile conical tube placed on ice, centrifuged at 800g and the supernatant divided into small aliquots and stored at -80°C until assayed. The pellet of cells were also stored at -80°C until assayed, however, due to low recovery of antigen specific T-cells, these were of poor quality and low in numbers which were not useful for analysis. No vaginal biopsies were collected during this study due to the concern of scarring which could affect challenge outcome.

Rhesus IFN-gamma Enzyme-linked immunospot assay (ELISpot)

Interferon-gamma (IFN-γ) ELISpot was performed as previously described for macaque [54] to determine antigen specific (IFN-γ) secreting cells from immunized animals. Cells were stimulated overnight in the presence of either specific peptide antigens (SIV-1mac239 Gag or Pol (NIH AIDS Research & Reagent Program, Germantown, MD) and SIVsmE660 Env (Invitrogen), R10 (negative control), or Concanavalin A (positive control).

Detection of SIV-specific antibodies

The presence of antibodies specific for SIV antigens was semiquantitatively determined by WB. The IgA and IgG SIV-specific antibodies from sera and genital secretions were analyzed using SIV western blot strips from ZeptoMetrix Corp (Buffalo, NY). Strips were incubation overnight with dilutions of sera or vaginal secretions normalized to ~0.5 μg IgG or IgA/ strip. The WB strips were developed with affinity purified alkaline phosphatase-conjugated goat anti-monkey IgA and with peroxidase-conjugated goat anti-monkey IgG reagents (Rockland Immunochemicals, Pottstown, PA). The reactivity of samples with particular SIV antigens was visualized after the addition of alkaline phosphatase (Bio-Rad, Hercules, CA) and peroxidase (Sigma, St. Louis, MO) substrates. The densities of relevant bands of assay samples were measured using an AlphaImager 3400 (Alpha Inotech Corp, San Leandr, CA). According to the intensity of the resulting blue and red bands to a particular SIV antigen, arbitrary values ranging from 0 to 4 were ascribed to each sample. For SIV Envelope- specific antibodies endpoint titers were determined as previously reported[55].

V1/V2 mapping

V1/V2 mapping was performed by using peptides for the V1/V2 region of SIVsmE660 Envelope region. Nunc MaxiSorp (Rochester, NY) plates were coated with approximately 1 Yg/ml pooled peptides. Plates were blocked with 10% fetal bovine serum followed by washing in 0.1% polysorbate 20 in PBS. Serum was diluted 1:50. Plates were washed and an anti-monkey IgG HRP secondary antibody (SouthernBiotech, Birmingham, AL) was added. Plates were washed and developed using the Sigmafast OPD substrate (Sigma). Values are reported as the OD read at 450 nm.

Determination of neutralizing antibody titers

Neutralizing antibody responses against tier 1 SIVsmE660.11 were measured using luciferase-based virus neutralization assays with TZM-bl cells as previously described[56].

Antibodies for PBMC flow cytometry

Surface stain monoclonal antibodies (mAbs) include: anti-CD4 [L200], anti-CD49d α4 integrin [9F10] and anti-CD95 [DX2] (BD Biosciences, San Jose, CA); anti-CD14 [TUK4], anti-CD20 [HI47] and LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen, Grand Island, NY); anti-CD28 [CD28.2] (Beckman Coulter, Pasadena, CA); anti-CD8 [2ST8.5H7] (Custom, mAb from Serotec, conjugation kit from Invitrogen).Intracellular stain mAbs include: anti-CD3 [SP34-2] and anti-TNF-α [MAb11] (BD Biosciences); anti-IFN-γ [4S.B3] and anti-IL-2 [MQ1-17H12] (Biolegend, San Diego CA).

Flow cytometry staining protocol for PBMCs

PBMCs were isolated from RhMs and cryopreserved. Samples were thawed and stimulated overnight (18 hours) in R10 at 2×10^6 cells/mL with: (i) SIVmac239 peptide pools specific for Gag or Pol, R10 (negative) or Staphylococcal Entertoxin B (SEB, positive). 1ul/mL GolgiPlug (brefeldin A) and 0.7ul/mL GolgiStop (monensin) (BD Biosciences) were added 1 hour after stimulation began. Cell were then stained as previously described[13].

Intravaginal challenge of rhesus macaques

All 28 animals were intravaginally challenged with 500 $TCID_{50}$ SIVsmE660 prepared in the laboratory of Dr. Phil Johnson (Children's Hospital of Pennsylvania) twice a week for 2 weeks. The $TCID_{50}$ of this stock was re-titered in CEMx174 cells at the time of challenge and was 4000 $TCID_{50}$. The dose was chosen to mimic early HIV infection. Depo-Provera was not used during the challenge to increase the RhMs ability to become infected. Blood samples were collected twice weekly for 6 weeks, weekly for 2 weeks, and then monthly to day 190 after challenge to monitor plasma viral load. TRIM5α analysis was performed and did not affect challenge outcome. Additional blood and tissue samples were collected at days 14, 28, and 56 after challenge and processed as described for the pre-challenge samples. RhMs were defined as aborted infections if the viral loads remained below the level of detection for the remainder of the study (6 months post challenge). Time to viral control was determine as the number of days after initial infection to the first day viral loads were undetectable or 150 days if progressively infected.

SIV viral RNA quantitation

SIV viral RNA was quantitated using a procedure described previously[57,58].

Statistical analysis

Data are presented as the mean ± S.E.M or median as specified in the figure legends based off of the normalcy of the data as calculated from triplicate wells from each experimental group. The statistical difference between immunization groups was assessed by using Mann-Whitney test, modified ANOVA test or fisher exact test. Comparisons between samples with a *P* value -0.05 were considered to be statistically different and therefore significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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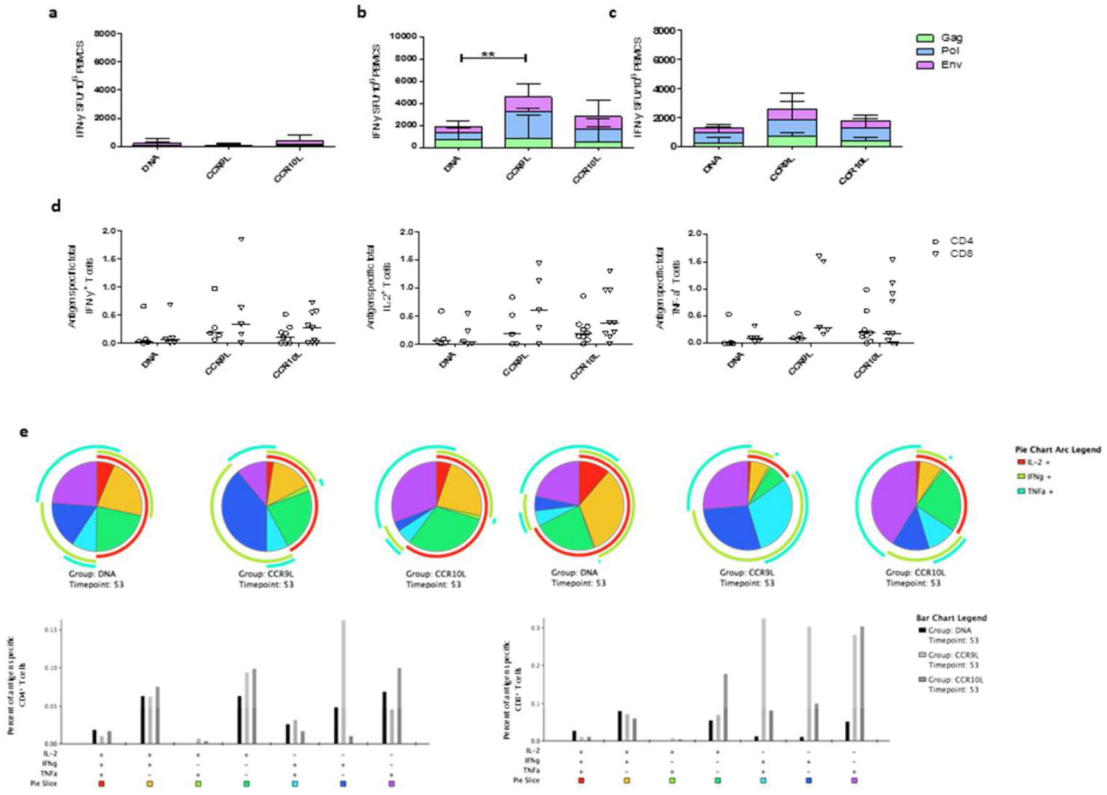

Fig. 1. Cellular response induced by vaccination

Interferon-gamma secreting cells against Gag (green) Pol (blue) and Env (purple) were enumerated by ELISpot assay. Responses were measured at week 0 (**a**) week 20 after 4th vaccination (**b**) and week 53 recall / final immunization (**c**). Intracellular cytokine staining was performed on cells stimulated with peptides from Gag and Pol and totaled for CD4 and CD8 at week 53(**d**). Polyfunctionality of PBMCs isolated at week 53 was determined for both CD4 (left) and CD8 (right) against Gag and Pol (**e**). Bars indicate median with interquartile range and the *P*-value reported for week 20 total IFN-γ Spot forming units (SFU) was calculated using the Mann-Whitney test.

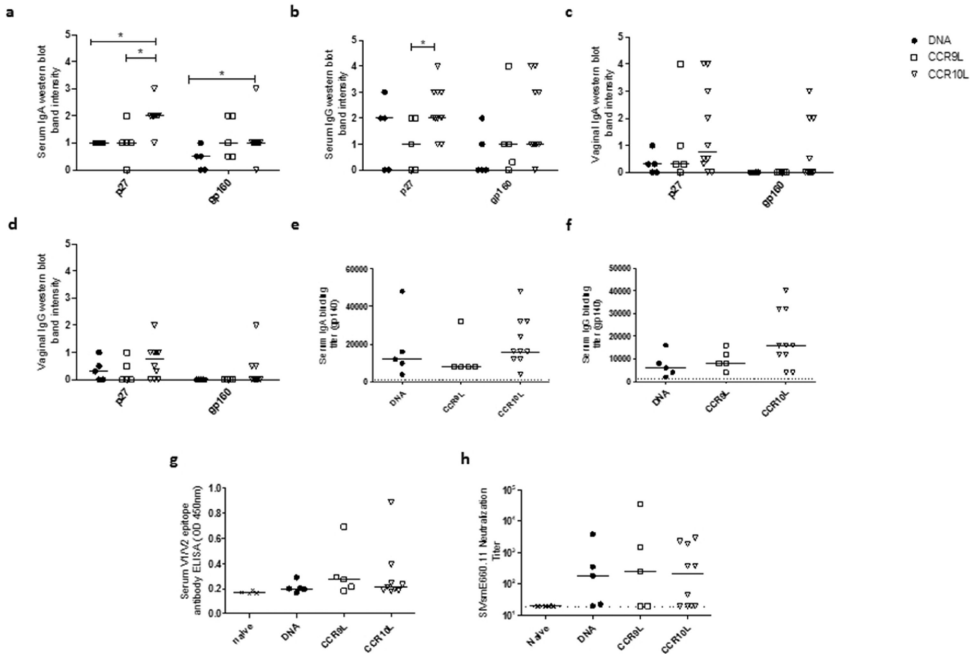

Fig. 2. Chemokine adjuvants enhance the vaccine-induced humoral response

Antibody responses were measured at week 50 (2 weeks after final vaccination). Serum IgA (**a**) and IgG (**b**) antibodies specific to Gag (p27) and Envelope (gp160) expressed as WB band intensities. Serum IgA against p27 was elevated in the group received CCR10L compared to DNA only ($P < 0.05$) and CCR9L ($P<0.05$). IgA (**c**) and IgG (**d**) against Gag (p27) and Envelope (gp160) measured in Ig-normalized vaginal secretions and expressed as WB band intensities. Serum IgA (**e**) and IgG (**f**) antibodies against gp140 Envelope protein expressed as ELISA endpoint titers. Serum IgG antibody binding to 15mer peptides spanning the V1/V2 region of Envelope (**g**). SIVsmE660.11 serum antibody neutralizing titers measured by TZM-bl assay (**h**). Dashed lines denote the limit of detection for respective assay. Bars indicate median. The *P*-values reported were calculated using the Mann-Whitney test. * indicates a $P < 0.05$.

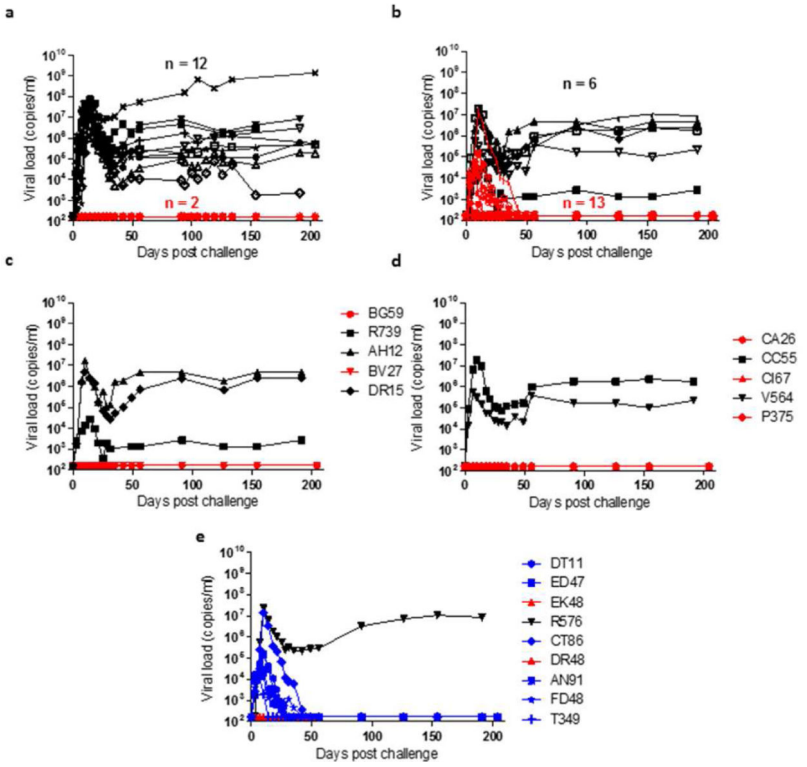

Fig. 3. DNA vaccination and chemokine adjuvants improve the challenge outcome

Animals were intravaginally challenged twice a week with SIVsmE660 26 weeks after the final booster vaccination and the viral loads were determined. The color black indicates animals with progressive infection, blue with abortive infection, and red the uninfected animals. Viral load in (**a**) vaccine-naïve animals (n=14); (**b**) all vaccinated animals (n=19); (**c**) in DNA only vaccinated animals (n=5); (**d**) in CCR9L vaccinated animals; and (**e**) in CCR10L vaccinated animals (n=9).

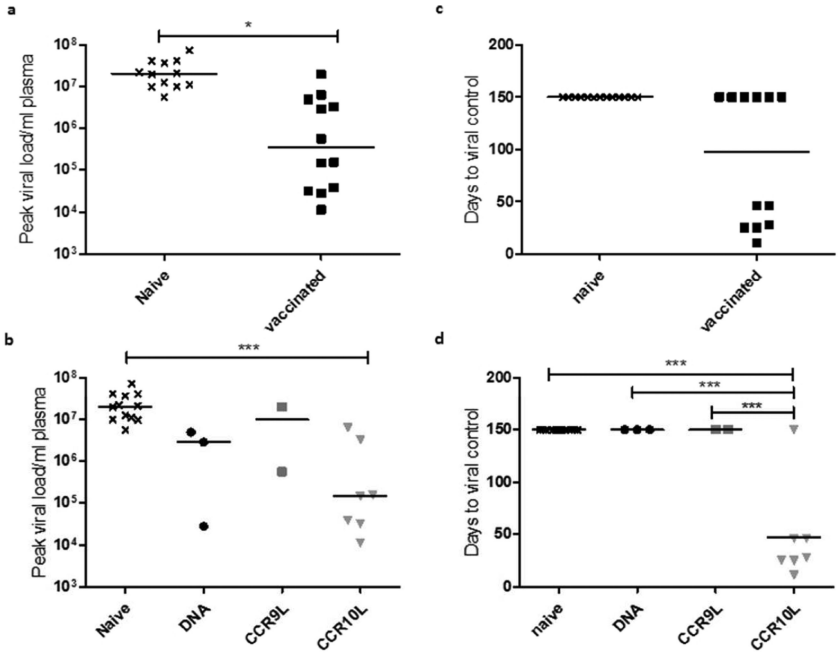

Fig. 4. CCR10L adjuvanted vaccine improved the challenge outcome characteristics

(**a**) Peak viral loads in animals that became infected. The peak of viral load was significantly (P<0.05) reduced in vaccinated compared to naïve animals. (**b**) Peak viral loads of each group of vaccinated animals. Peak viral loads was significantly reduced (*P*<0.05) in RhMs receiving CCR10L adjuvanted vaccine compared to naïve animals. (**c**) The number of days until viremia reached undetectable levels in infected RhMs. Animals in which viremia was never controlled were scored as day 150. Animals receiving CCR10L adjuvant had a shorter time to control of viremia compared to animals receiving CCR9L adjuvanted vaccine (*P*<0.001), DNA only (*P*<0.001) and naïve animals (*P*<0.001). Bars indicates mean. The *P*-values reported were calculated using the Student-T test for (a) and a modified ANOVA for (b) and (c).

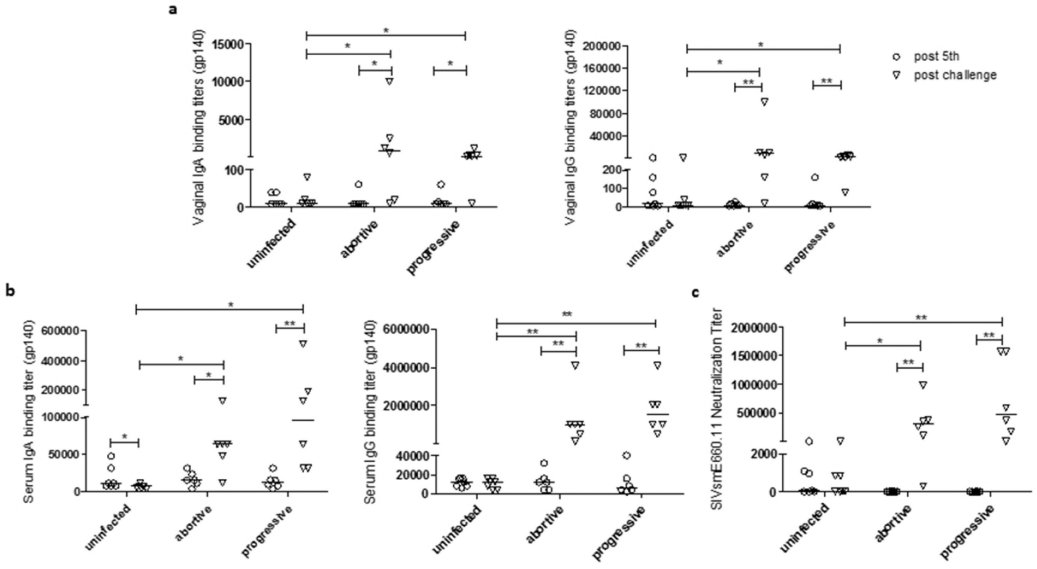

Fig. 5. Differential induction of humoral responses post challenge

Humoral responses were monitored after last immunization and at two months post challenge. Serum IgA and IgG specific for gp140 Envelope glycoprotein in vaginal secretions (**a**) and serum (**b**) expressed by ELISA endpoint titers. Neutralization titers against SIVsmE660.11 after final vaccination and at two months post challenge (**c**). RhMs were assigned to either uninfected, aborted or progressively infected groups based on the challenge outcome. Bars indicate median. The *P*-values reported were calculated using the Mann-Whitney test. * indicates a $P < 0.05$ and ** indicates a $P < 0.01$.

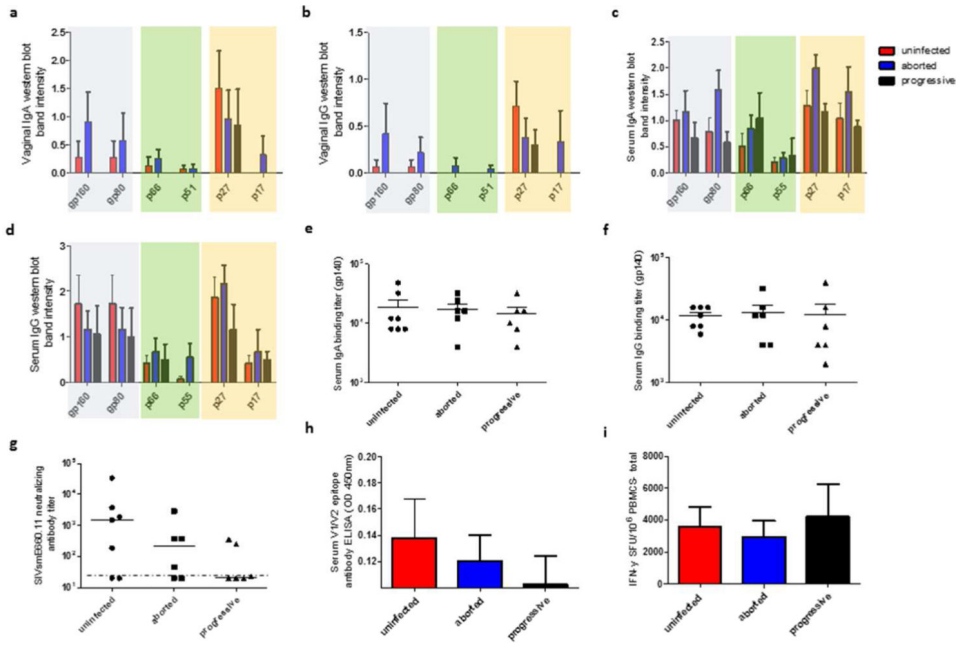

Fig. 6. Correlates analysis of humoral and cellular responses

Antigen specific antibody and IFN-γ responses were measured 2 weeks following the final vaccination. Vaccinated animals were grouped according to their challenge outcome in: uninfected (n=7) (red), aborted (n=6) (blue) and progressive infection (n=6) (black) groups. Vaginal IgA (**a**) and IgG (**b**) antibodies specific for different HIV proteins, expressed as intensity of WB bands. Both vaginal IgA and IgG antibodies were elevated in uninfected and aborted infection compared to progressively infected animals. Serum IgA (**c**) and IgG (**d**) for different HIV proteins, expressed as WB band intensity. Serum IgA (**e**) and IgG (**f**) against SIV gp140, expressed as ELISA endpoint titers. Antibody neutralizing titers (**g**) against SIVsmE660.11 isolate were elevated in uninfected animals compared to abortive and progressive infection. Serum IgG (**h**) antibodies binding to V1/V2 were elevated in uninfected, compared to progressively infected animals. Total IFN-γ (**i**) responses to Gag, Pol, and Env by peripheral blood mononuclear cells (PBMCs). The number of IFN-γ secreting cells was determined by ELISpot assay and expressed as spot forming units (SFU). Dashed lines denote the limit of detection for respective assay. Bars indicates mean with s.e.m.