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Systemic Immunization with CCL27/CTACK Modulates Immune Responses at Mucosal Sites in Mice and Macaques

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Abstract

Plasmid DNA is a promising vaccine platform as it has been shown to be safe and able to be administered repeatedly without vector interference. Enhancing the potency of DNA vaccination through co-delivery of molecular adjuvants is one strategy currently under investigation. Here we describe the use of the novel chemokine adjuvant CCL27/CTACK to enhance immune responses to an HIV-1 or SIV antigen in mice and rhesus macaques. CCL27 has been shown to play a role in inflammatory responses through Chemotaxis of CCR10+ cells and we hypothesized that CCL27 may modulate adaptive immune responses.

Immunizations in mice with HIV-1 gag/CCL27 enhanced immune responses both at peripheral and surprisingly mucosal sites. To confirm these findings in a large animal model, we created optimized CCL27 and SIV antigenic plasmid constructs for rhesus macaques. 10 macaques (n=5/group) were immunized intramuscularly with 1mg/construct of antigenic plasmids +/- CCL27 with electroporation. We observed significant IFN- γ secretion and CD8+ T cell proliferation in peripheral blood. Interestingly, CCL27 co-immunized macaques exhibited a trend toward greater effector CD4+T cells in the bronchiolar lavage (BAL). CCL27 co-delivery also elicited greater antigen specific IgA at unique sites including BAL and fecal samples, but not in the periphery. Future studies incorporating CCL27 adjuvant in vaccine or therapy models where eliciting immune responses in the lung are warranted.

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Introduction

Enhancing the potency of cellular and/or humoral immune responses generated by DNA vaccines for HIV-1 is a critical focus of the field. In addition to improved delivery techniques, enhanced construct design, and heterologous prime-boost strategies, the use of molecular adjuvants are strategies employed to augment DNA vaccine elicited immune responses [1-5]. Molecular adjuvants such as chemokines and cytokines can be incorporated into a vaccine strategy to skew the immune response towards cellular or humoral immunity as well as alter the magnitude and duration of the elicited response [6-15].

In this study we evaluated for the first time the efficacy of a novel potential adjuvant Cutaneous T cell Attracting Chemokine (CTACK), or CCL27, to modulate immune responses when delivered as a plasmid-encoded DNA vaccine with electroporation. CCL27 is secreted from skin keratinocytes [16-19] and has been shown to attract cutaneous lymphocyte antigen (CLA) positive cells expressing the cognate receptor CCR10 [17, 20]. In addition, skin-derived Langerhans cells have also been shown to express CCR10 [20] as well as IgA antibody secreting B cells (ASC) [21]. CCL27 appears to play an important role in inflammation, with enhanced serum levels observed in diseases such as atopic dermatitis and psoriasis [22, 23]. The increase of CCL27 leads to the enhanced recruitment of CCR10+ cells and together they have been shown to play an important role in T cell mediated skin inflammation [24]. We have previously reported intramuscular immunization with CCL27 and influenza hemagglutinin augmented antigen specific IgA and T cell responses that protected mice from a lethal challenge [25]. Interestingly, CCL27 has also been shown to be upregulated in the lungs of macaques infected with tuberculosis [26] supporting a role for CCR10 in immune localization to this mucosal site. Because CCL27 appears to play an important role in recruiting lymphocytes and causing an inflammatory response in the lung, we were interested to see if the inclusion of CCL27 could adjuvant immune responses when co-delivered in a DNA with electroporation vaccine strategy in non-human primates.

We report here that CCL27 is a unique adjuvant when co-delivered as a DNA plasmid in mice and macaques. CCL27 with an antigenic HIV-1 or SIV gag plasmid slightly enhanced immune responses at unique immune sites in both mice and macaques. In mice we observed an increase in antigen specific IFN- γ secreting cells and IgA in both peripheral (spleen, sera) and mucosal compartments (mesenteric lymph node, (MLN), fecal pellets). In a pilot macaque study, immunization with pCCL27 and SIV antigens modulated IgA at mucosal sites including the bronchial lavage (BAL) and in fecal samples as well as enhanced T cell responses in the lung. Co-delivery of pCCL27 did not adjuvant immune responses detected in the periphery, as was observed in mice. These results suggest that delivery of a chemokine in a systemic immunization may modulate immune responses at some sites. Further study of this strategy for enhancing immune responses at specific infectious target sites is warranted.

Materials and Methods

Plasmid Preparation

For mouse studies, the HIV-1 consensus B (pHIV-1gag) was prepared as previously described [27]. Cloning of murine CCL27 (NM011336) into the pVAX1 vector was carried out following generation of the chemokine cDNA from RNA extracted from murine ear, and verified by sequence analysis. The SIV DNA constructs encoding consensus SIV Gag (pGag), SIV Pol (pPol), and SIV Env (pEnv) were generated in our laboratory [28]. Modifications were performed to each antigenic sequence including the addition of an IgE leader to improve expression. In addition, a constitutive transport element was added to Gag, and seven mutations were introduced into Pol to deactivate the protease, reverse transcriptase, RNase H, and integrase regions. The N-linked glycosylation sites in the V1 and V2 loops of Env were removed and the cytoplasmic tail was truncated to prevent envelope recycling. These modified SIV constructs as well as the rhesus CCL27 construct (pCCL27) were subsequently RNA and codon optimized to further improve expression, and cloned into the pVAX1 expression vector by GENEART (Toronto, ON). These constructs were then sent to VGX Pharmaceuticals (The Woodlands, TX) for large-scale production.

Mouse Studies

The quadriceps muscles of 6 to 8 week old female Balb/C mice (Jackson Laboratory, Bar Harbor, ME) were injected three times, two weeks apart, with plasmid DNA formulations containing combinations of vector backbone (pVAX, Invitrogen, Carlsbad, CA), antigen pHIV-1gag (50ug), and murine CCL27 (100ug) as described in previous publications [27, 29]. Formulations contained in 0.25% bupivacaine-HCL (Sigma, St. Louis, MO) in isotonic citrate buffer. All mice were housed in a temperature-controlled, light-cycled facility at the University of Pennsylvania, and their care was under the guidelines of the National Institutes of Health and the University of Pennsylvania.

Macaque Studies

A group of rhesus macaques (n=5 per group) were immunized at weeks 0, 6, 12, and 18 with 1mg per construct of pSIVgag, pSIVpol, pSIVenv only (DNA group) or with 1mg pCCL27 (CCL27 group). DNA was formulated in water with 1% low molecular weight poly-L-glutamate sodium salt [30] and delivered into the quadriceps muscle followed by *in vivo* electroporation using the constant current CELLECTRA® device (VGX Pharmaceuticals) [31]. Rhesus macaques (*Macaca mulatto*) were housed at BIOQUAL, Inc. (Rockville, MD) 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 in quarantine prior to any immunization.

In-vitro Translation assay

The TNT- T7 Quick Coupled Transcription/Translation Reticulocyte Lysate system (Promega, Madison, WI) and [³⁵S] methionine were used to create labeled rhesus CCL27 protein product. pVAX vector alone (negative control) or pVAX vector encoding CCL27 (pCCL27) and [³⁵S] methionine were added to the reaction mix according to the

manufacturer protocol. The reaction was carried out at 30°C for 1 hour. Labeled proteins were immunoprecipitated using 5µg purified monoclonal anti- human CCL27 antibody (R&D Systems, Minneapolis, MN) at 4°C with rotation overnight in RIPA buffer. Approximately 5mg of protein G-Sepharose beads (Amersham Biosciences, Buckinghamshire, United Kingdom) was added to each immunoprecipitation reaction, and the samples were incubated at 4°C with rotation for 2 hours. The beads were washed three times with binding buffer containing high salt and bovine serum albumin and finally suspended in 2× sample buffer. The immunoprecipitated protein complexes were eluted from sepharose beads by boiling for 5 minutes and were run on a 12% SDS-PAGE gel (Cambrex, Rockland, ME). The gel was fixed, treated with amplifying solution (Amersham Biosciences), and dried for 2 hours in a gel drier (Bio-Rad, Hercules, CA). The dried gel was exposed to X-ray film at -80°C and developed using the Kodak automatic developer (Kodak, Rochester, NY).

Transfections

Expression levels of the CCL27 plasmid construct were tested following transient transfection of RD cells (ATCC, Rockville, MD). Cells were plated in 60×15mm tissue culture dishes at a density of 2.5×10^5 cells per well in complete DMEM plus 10%FBS (BRL, GIBCO) and allowed to adhere overnight in a 37°C incubator. The next day cells were transfected with control pVAX or pCCL27 (3µg/well) using FuGENE 6 Transfection Reagent (Roche Diagnostics) according to manufacturer's protocol. After forty-eight hours, cell supernatants were harvested and analyzed for the presence of CCL27 protein by commercial ELISA kits (R&D systems).

Blood collection- Macaque

Animals were bled every two weeks. 10 or 20mL of blood was collected in EDTA tubes, and peripheral blood mononuclear cells (PBMC) were isolated by standard Ficoll-hypaque centrifugation and re-suspended in complete culture R10 medium (RPMI 1640 with 2mM/L L-glutamine, 10% heat-inactivated fetal bovine serum, 100IU/mL penicillin, 100µg/mL streptomycin, and 55µM/L β-mercaptoethanol). Red blood cells (RBC) were lysed with ammonium chloride-potassium (ACK) lysis buffer (Cambrex BioScience, East Rutherford, NJ).

Processing of Fecal Pellets

Fecal pellets were weighed (on average 1-5 grams was retrieved) and 1mL/gram of a 0.01% BSA, 0.02% Sodium Azide solution containing protease inhibitor tablets (Roche, Indianapolis, IN) was added. After vigorous vortexing (~15 minutes), samples were centrifuged (1200 rpm for 20 minutes) and the supernatant transferred to a clean tube for further high-speed centrifugation (16,000g for 15 minutes). The resulting supernatant was filtered, aliquoted, and stored at -80°C.

Bronchiolar lavage (BAL)

Bronchiolar lavages were performed according to protocol. Briefly, a catheter was put into the airway of the anesthetized animal, and a total of 75ml was flushed through the catheter

in 15ml increments. BAL fluid was centrifuged to pellet lymphocytes and the fluid was stored at -80 until analysis.

Enzyme-linked immunospot assay (ELISpot)

ELISpot assays were conducted as previously described for mouse [29] and macaque [32]. Briefly, ELISpot 96-well plates (Millipore, Billerica, MA) were coated with anti-mouse (R&D systems, Minneapolis, MN) or anti-human [clone GZ-4] (Mabtech, Cincinnati, OH) interferon-gamma (IFN- γ) capture antibody and incubated overnight at 4°C. The following day, plates were washed with PBS and blocked for 2h with R10. 2×10^5 mouse splenocytes or macaque PBMCs (1×10^5 at later time points) from each group were added per well and stimulated overnight at 37°C in the presence of R10 (negative control), HIV-1 consensus Clade B Gag or SIVmac239 Gag, Pol or Env peptide pool antigens (NIH AIDS Research & Reference Reagent Program, Germantown, MD). Peptide pools consist of 15-mer peptides overlapping by 11 amino acids. After 24h of stimulation, the cells were washed and incubated overnight at 4°C with anti-mouse (R&D) or anti-human IFN- γ biotinylated detector antibody [Clone 7-B6-1] (Mabtech). Plates were washed, and streptavidin-alkaline phosphatase (R&D Systems, Minneapolis, MN) was added to each well and incubated for 2h at room temperature. After washing, BCIP/NBT chromogen (R&D Systems) was added. The plate was then rinsed with water and dried. Spots were counted by an automated ELISpot reader (CTL Limited, Shaker Heights, OH) and antigen-specific responses were determined by subtracting the number of spots in the negative control wells from the antigen-stimulated wells.

CFSE Staining

PBMCs were stained with 5 μ M carboxyfluorescein diacetate, succinimidyl ester (CFDA SE) (Invitrogen) for 10 minutes at 37°C. Cells were then washed with R10 media and resuspended in a concentration of 10×10^6 cells per mL. 100 μ L of cells were plated in a 96-well round bottom plate with 100 μ L of each antigenic stimulator containing SIV peptide pools (1:200) with 2 μ g/mL recombinant SIV p27 or gp130 (ImmunoDiagnostics, Woburn, MA) added to the appropriate wells. For controls, R10 (negative), and 5 μ g/mL Concavalin A (positive) were used. After 5 days in culture, cells were washed and stained with a LIVE/DEAD Violet stain (Invitrogen) according to manufacturer's protocol for 10 minutes at 37°C. Cells were then washed and stained for surface markers using the following antibodies from BD Biosciences (San Jose, CA) CD3-APC-Cy7 [Clone SP34-2], CD4-PerCP-Cy5.5 [L200], CD8-APC [SKI], CD14-PacBlue [M5E2], CD16-PacBlue [3GB], and CD20-PacBlue [2H7] (eBiosciences, San Diego, CA) for 30 minutes at 4°C. Cells were then washed two times and fixed in 1% paraformaldehyde (PFA) in PBS. Data was collected on a LSRII flow cytometer (BD Biosciences, Franklin Lakes, NJ) and analyzed using FlowJo software (Tree Star, Ashland, OR). At least 30,000-50,000 live CD3+ T cells were collected and values from the R10 stimulation alone were subtracted from antigenic stimulation groups prior to graphing.

Enzyme-linked immunosorbant assay (ELISA)

To determine sera antibody titers against HIV Gag (mouse) or SIV Env (macaque), 96-well high-binding polystyrene plates (Corning, Lowell, MA) plates were coated overnight at 4°C

with 100 ng/well of recombinant HIV p24 or SIV gp130 protein (Immunodiagnostics) or BSA (control) diluted in PBS. The next day, plates were washed with PBS, 0.05% Tween 20 (PBST), blocked for 1h with 3% BSA/PBST, and incubated with serial dilutions of serum from immunized animals overnight at 4°C. Bound IgG or IgA was detected using goat anti-mouse IgG or IgA (Santa Cruz, Santa Cruz, CA) or goat anti-macaque IgG-HRP or IgA-HRP (Nordic) at a dilution of 1:10,000 or 1:8,000. Bound enzyme was detected by the addition of the chromogen substrate solution TMB (R&D Systems), and read at 450nm on a Biotek (Winooski, VT) EL312e reader. Endpoint titer is defined as the highest dilution of sera at which the O.D. values against Env remain two-fold over corresponding BSA values. For mouse, the amount of total IgG or IgA in sera or fecal secretions was calculated by interpolating the optical densities on calibration curves, using the DeltaSoft II program (BioMettalics, Inc., Princeton, NJ).

Intracellular Cytokine Staining

Lymphocytes retrieved from the bronchiolar lavage (BAL) of macaques rested overnight at 37°C in R10 media prior to stimulation. The rested BAL and fresh PBMCs from rhesus macaques were stimulated for 5-5.5 hours with either SIVmac239 peptides, R10 (negative) or Staphylococcal Enterotoxin B (SEB) (positive) in the presence of 1μl/mL of golgi transport inhibitors GolgiStop and GolgiPlug (BD Biosciences). During the incubation, CD107a-FITC (BD Biosciences) was also included. Following stimulation, cells were stained for viability and extracellular markers as in the T-cell proliferation assay. However CD4-PE-Cy5.5 [OKT-4] (eBiosciences, San Diego, CA) was used in addition to CD28-ECD [CD28.2] (Beckman Coulter, Fullerton, CA) and CD95-PE-Cy5 [DX2] (BD Biosciences). After washing, cells were fixed and permeabilized according to protocol using Cytofix/Cytoperm (BD Biosciences) and stained for intracellular expression of CD3-APC-Cy7, IFN- γ -Alexa700 [SP34-2], TNF- α -PE-Cy7 [Mab11], and IL-2-PE [MHQ1-17H12] (all from BD Biosciences). Murine MLN lymphocytes were harvested from individual mice (n=4), stimulated with HIV-1 gag peptides, and stained similarly except antibodies included a FcR block (CD16/CD32), CD4-Alexa700, CD8-PerCP, CD3-PECy5 and IFN- γ PE-Cy7 (all from BD biosciences). Cells were then washed, fixed, run, and analyzed similar to CFSE. At least 50,000-100,000 live CD3+ T cells were collected and background responses in the media wells were subtracted from each stimulation group.

Results

Immunization with CCL27 enhances HIV-1 gag specific IFN- γ secretion and IgA in mice

To determine whether co-delivery of plasmid-encoded CCL27 (pCCL27) could modulate immune responses in a DNA with electroporation vaccine strategy groups of BALB/c mice were immunized in the tibialis anterior (TA) muscle with an empty vector control (pVAX), 50 μg of an antigenic HIV-1 gag plasmid (pHIV-1gag), or 50 μg of HIV-1 gag and 100 μg of pCCL27 (pCCL27) three times, two weeks apart. One week following the final immunization, samples were harvested for both cellular and humoral immune analysis. As shown in Figure 1A, splenocytes from pCCL27 co-immunized mice had a modest increase in IFN- γ secretion when compared to the pHIV-1gag immunized group alone as measured by ELISpot (450 vs 300 spot forming cells (SFCs)/million splenocytes). Lymphocytes from

the mesenteric lymph node (MLN) of pCCL27 immunized mice showed a more dramatic 4-fold increase in IFN- γ secretion over the antigen group alone as measured by intracellular cytokine staining (ICS) (Figure 1B).

The ability of CCL27 to augment humoral immune responses was also determined. Co-vaccination with pCCL27 resulted in higher gag-specific IgA found in both the sera (Figure 1C) and fecal (1D) samples of immunized mice, suggesting that the inclusion of the chemokine may be altering mucosal as well as systemic immune responses. Levels of IgG were similarly enhanced (data not shown). Since it may be possible that the observed IgA in sera and fecal samples may be the result of transudation, a B-cell ELISpot to detect IgA antibody secreting cells (ASCs) was performed. Splenocytes (Figure 1E) or gut biopsy collected tissue (Figure 1F) of immunized mice was analyzed for HIV-1 gag specific IgA. Interestingly, levels of HIV-1 gag specific IgA were statistically higher in co-immunized mice in both samples, supporting the idea that CCL27 may be adjuvanting this response. This data supports previous data utilizing intramuscular immunization (without electroporation) and influenza HA antigens [25]. As many adjuvant molecules have been reported to improve responses in mice, and then were later shown to lack such activity in human studies, we sought to further study this DNA vaccine adjuvant in the important rhesus macaque model as an important comparison.

Co-delivery of CCL27 augments cellular immune responses in the lung, not the periphery in rhesus macaques

An SIV adjuvant construct, CCL27, was codon and RNA optimized for rhesus macaques and cloned into the pVAX1 expression vector as described in the materials and methods section (Figure 2A). Expression of the construct was confirmed by an *in vitro* translation assay (Figure 2B). Supernatants from cells transfected with pCCL27 or the empty pVAX1 vector were run on a sandwich ELISA to detect the CCL27 protein (2C). After confirming expression of the CCL27 protein, rhesus macaques (n=5) were immunized with optimized plasmids encoding consensus SIV Gag, Pol, Env (DNA group) [28] or the same SIV antigenic plasmids and CCL27 (CCL27 group). Rhesus macaques were given a total of four intramuscular immunizations with electroporation (Table 1) and blood samples were collected approximately every two weeks.

Immune responses elicited by intramuscular immunizations were measured in peripheral blood by a standard IFN- γ ELISpot assay to detect the number of SIV antigen specific IFN- γ secreting cells (Figure 3A). Peripheral blood mononuclear cells (PBMCs) were isolated from blood 2 weeks post each immunization. Robust immune responses were detected after 2 immunizations with an average of 4, 228 and 5, 347 IFN- γ SFC/10⁶ PBMCs for DNA and CCL27 respectively. These responses were nearly doubled following the third immunization to 8,838 SFCs for DNA and 9, 478 SFCs for CCL27. Following the fourth immunization, responses reached impressive levels: 14, 737 and 11, 322 SFCs for DNA and CCL27 respectively. While the immune responses elicited by DNA immunization were quite high, the addition of CCL27 did not appear to further enhance the observed responses by the final immunization.

It is likely that the proliferative capacity of vaccine-induced effector cells will be important in combating viral infection. Accordingly, the proliferative potential of the generated SIV specific T cells in the peripheral blood was examined. Using CFDA-SE to label cells, several rounds of cell division was detected after culture for 5 days with peptide and protein stimulation (Figure 3B,C). In particular, CD8+ T cells from both the DNA and the CCL27 vaccinated groups had similar high levels of proliferation (18% and 16 %, respectively) following the fourth immunization (Figure 3C).

Cytokine expression by group was next assayed by ICS. Figure 4 shows the total magnitude of cytokine expressing CD4+ and CD8+ T cells in response to stimulation with SIV peptides from the vaccine antigens. The total CD4+ T cell response was approximately 1% in both DNA and CCL27 immunized macaques (Figure 4A) whereas the CD8+ T cell response was nearly 4% in DNA and less in the CCL27 group. The majority of the cytokine response in both T cell subsets was in response to Env stimulation. Figure 4B shows the single function cytokine profile of responding cells to all antigens for both CD4+ and CD8+ T cells. This data supports the previous two findings suggesting that CCL27 has little adjuvanting effect on antigen specific immune responses in peripheral blood. However, we next decided to look at lymphocytes from the bronchiolar lavage (BAL) as previous reports have shown an upregulation of CCL27 in the lungs of macaques during severe tuberculosis infection [26]. BAL lymphocytes were assayed for cytokine expression and revealed a striking 2-fold enhancement in the CCL27 co-immunized group immune response (Figure 5). As CCR10, the receptor for CCL27 is found primarily on CD4+ T cells, we looked at the CD4+T cell responses in the BAL. The limitations of cell number limited the examining of response against only one antigen. Therefore we chose to look at SIV Env as it gave the best response in peripheral blood. We found an enhanced expression the effector functions: 107a (a marker of degranulation) as well as IFN- γ , in CD4+T cells from the lung in CCL27 immunized macaques, whereas these levels were similar in the PBMCs from CCL27 immunized macaques and DNA group (Figure 5). Somewhat unexpectedly, the DNA immunized group even had low levels of cytokine secretion in BAL CD4+ T cells. These data suggest that CCL27 may be driving cellular immune responses at this important immune site.

To determine whether the observed peripheral responses in CCL27 immunized macaques were similar to those in the DNA group over time, we looked at immune responses in the blood 23 weeks following the final immunization (Figure 6). Memory responses as measured by IFN- γ ELISpot (Figure 6A, B) and CD8+ T cell proliferation (Figure 6C) show the vaccine-elicited responses were persistent. The averages of IFN- γ SFC/10⁶ PBMCs dropped to levels similar to those observed following the 2nd immunization: 4,334 in the DNA group and 5,345 in the CCL27 group (Figure 6B). Levels of CD8+ T cell proliferation, on the other hand, remained similar to those observed following the final immunization (approximately 18% in the DNA and 13% in the CCL27 group) (Figure 6C).

Co-delivery of CCL27 modulates IgA responses in rhesus macaques

As CCL27 appeared to be influencing cellular responses in the lung, but not the periphery, we wanted to next see whether the humoral immune response would be affected as well. We

began by examining the levels of IgG and IgA antibodies in the sera of immunized macaques. As shown in Figure 7A, the levels of IgG antibodies specific for SIV Env in the sera of DNA and CCL27 immunized macaques both peak at 1:1,000, with no difference between the groups. Similarly, levels of Env-specific IgA in the sera were not increased with co-delivery of CCL27, although one macaque in the DNA group exhibited an increase in sera IgA at the time point shown (10 weeks post the 4th immunization) (Figure 7B). To further characterize the CCL27 mediated induction of an immune response, we next measured antigen specific IgA from fecal extracts (Figure 7C) and collected from bronchiolar lavage (Figure 7D). In these samples, more CCL27 immunized macaques had titers of IgA specific for Env. In fecal samples, 4/5 CCL27 co-immunized macaques had titers of IgA compared to 0/5 in the DNA group (Figure 7C), whereas 2/5 macaques in the CCL27 group (compared to 0/5 in the DNA group) had titers of IgA in the bronchial lavage (Figure 7D). Taken together the humoral immune responses mirrored the observed cellular immune responses in that there are modest increases in antigen specific immune responses in CCL27 immunized macaques compared to DNA despite very similar responses in the periphery.

Discussion

The need for an HIV-1 vaccine has never been greater and in the wake of the Merck Ad5 STEP study, there is a focus on developing new and improved vectors and vaccine strategies [1, 33, 34]. Plasmid DNA vectors are attractive candidates as they can be repetitively administered without the generation of anti-vector immunity. However, historically these vaccines are not as immunogenic as viral vectors. Formulations, construct optimization (leader sequences, codon usage, etc), delivery methods (electroporation, etc), and co-delivery of molecular adjuvants are all techniques being employed to enhance the immune response to DNA vaccines. The inclusion of cytokines and chemokines along with other elements is an effective strategy to augment immune responses generated by the antigenic plasmid alone.

Here we describe the ability of CCL27 (CTACK) to adjuvant immune responses for an HIV-1 (mouse) or SIV (macaque) antigenic plasmid-encoded vaccine. In conjunction with its cognate receptor, CCR10, CCL27 has been shown to play an important role inflammation by attracting receptor positive cells to the site of CCL27 secretion, namely the skin. CCL27 has been shown to play a significant role in the development of inflammatory diseases such as atopic dermatitis and psoriasis. CCL27 is also reported to play a role in lymphocyte trafficking to the lung [26] and its receptor is found on most IgA producing B cells [21]. When co-delivered with an antigenic HIV-1 gag construct, plasmid-encoded adjuvant CCL27 immunized mice exhibited increased IFN- γ secretion from MLN lymphocytes and to a lesser extent T cells isolated from the spleen. We also observed enhanced levels of HIV-1 gag specific IgA in the sera and fecal samples of the CCL27 co-immunized mice. B-cell ELISpot was employed to detect IgA ASC in the spleen and GALT and in both tissues, levels of HIV-1 gag specific IgA were increased in CCL27 co-immunized mice over the HIV-1 gag immunized mice only.

The receptor for CCL27, CCR10, has previously been reported to be expressed on IgA secreting B cells, however the data presented here represents the first report that incorporation of the CCL27 ligand can adjuvant these cells through an immunization strategy in rhesus macaques. Macaques were immunized with either antigenic constructs alone (SIVgag, SIVenv, SIVpol) or with antigenic constructs along with CCL27. Following immunizations, both groups of animals had similar levels of robust immune responses detected in the peripheral blood (IFN- γ ELISpot, proliferation, cytokine secretion) as well as levels of both IgG and IgA antibodies. However, when we expand responses from lymphocytes from the BAL, CCL27 immunized macaques expressed a trend toward increased levels of IFN- γ as well as CD 107a, a marker for degranulation, suggesting increased effector cell induction at these immune sites. In addition, levels of IgA in fecal samples of CCL27 co-immunized macaques were statistically greater than levels in the antigen group alone ($p < 0.05$).

The modulation of vaccine-elicited immune response at such mucosal sites by a plasmid vaccine encoding a unique chemokine adjuvant delivered intramuscularly represents a novel observation warranting further discussion and study. Historically, the generation of mucosal immunity requires immunization at mucosal sites as it is thought that the resident dendritic cells differentially prime T cells to become either mucosal or peripheral responder cells. Environmental cues such as Vitamin A/Retinoic Acid[35] or Vitamin D/1,25(OH) $_2$ D3 received by the dendritic cell in the local lymphoid region during T cell activation, programs the T cell for mucosal or cutaneous homing respectively [36, 37]. However, both retinoic acid and 1,25(OH) $_2$ D3 have been shown to drive primarily immunosuppressive effects including suppressed Th1 responses, reduced T cell proliferation, and increased regulatory T cell populations [36, 37], thus alternative effector mechanisms may also be relevant in this context.

In addition, published evidence supports that mucosal immune responses can be elicited by systemically delivered vaccines. It has been reported in both mice and humans that co-delivery of some adjuvants with antigen to the skin induces humoral and cellular responses that can target to the mucosa [38-45]. Furthermore, the responses elicited were protective against mucosal challenge with live virus in the mouse model[38, 41]. The ability of skin DCs carrying antigen to migrate into mucosal immune-inductive sites may explain these findings [38]. As skin-derived Langerhans cells have been shown to express CCR10 [20], this could help to explain how delivering CCL27 intramuscularly could be enhancing immune responses at specific sites such as the lung. It may be possible that CCL27 enhanced expression in the draining lymph node following vaccination can attract CCR10+ DC that can then migrate to the regional lymphoid sites of the lung. It is likely that not all CCR10 positive cells that are recruited by the adjuvants and activated in the systemic compartment will end up in the mucosa. This limitation is due to the secondary requirement of expression of specific adhesion molecules along with the specific chemokine receptor for such mucosal trafficking.

Further work concentrating on the subset of cells that traffic in response to chemokine adjuvanted DNA approaches will be informative in this regard. The novel use of unique chemokines as DNA vaccine adjuvants has potential implications for the development of

vaccination strategies that require targeted immunity. Future studies incorporating CCL27 adjuvant in vaccine or therapy models where eliciting immune responses in the lung are warranted.

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Figure 1. Co-vaccination with murine pCCL27 enhances antigen specific IFN- γ and IgA antibodies in the periphery and mucosa

Mice were immunized intramuscularly 3 times, two weeks apart. Samples were harvested one week following the final injection from pVAX control, pHIV-1gag, or pHIV-1gag/pCCL27 immunized mice. A) Splenocytes were cultured overnight with R10 media (negative control), or HIV-1gag peptide pools 1 through 4. The bar represents the total number of HIV-1 gag specific IFN- γ spot forming cells (SFC) per million splenocytes after background subtraction. B) Lymphocytes from the mesenteric lymph nodes were harvested and stimulated with either R10 or HIV-1gag peptides. Cells were stained as described in

methods and 50,000 CD3+ live lymphocytes (as determined by the LIVE/DEAD stain) were collected. Total IFN- γ secreting CD4+ (grey bar) or CD8+ (black bar). Responses from the negative control wells were subtracted from the antigenic stimulations prior to graphing. C,D) The average fold increase of anti-HIVgag-specific IgA elicited by the chemokine CCL27 adjuvant over pHIV-1gag antigenic plasmid alone is shown in panel C (sera IgA) and panel D (fecal IgA). Data from 6 independent experiments was averaged and fold increases were determined. Levels of HIV-1 specific IgA was determined by an ELISA assay against HIV-1gag p24 protein. Quantitation of fecal and serum IgA ($\mu\text{g/ml}$) was determined using a recombinant IgA standard of known concentration as the standard curve, and fold increase values were determined. E,F) CCL27 enhances IgA by B lymphocytes in the spleen (E) and gut-associated lymphoid tissue (GALT) (F). Whole splenocytes (E) or Peyer's patch (F) cells were placed in an HIV-1gag p24 protein coated, 96 well ELISpot blocked plate and incubated for 5hrs. Secreted anti-HIV-1gag IgA by effector or memory B cells is captured, counted, and graphed as HIV-1-specific IgA Antibody Secreting Cells (ASC) per million splenocytes or Peyer's patch cells. Statistical differences are noted with ** ($p < 0.01$) as determined by student's t-test.

Figure 2. The rhesus CCL27 plasmid encodes functional chemokine

A) Rhesus CCL27 was RNA and codon optimized for expression in rhesus macaques and inserted into a pVAX1 expression vector. B) Plasmid CCL27 expresses the appropriate size chemokine (12.4 kDa) as detected by radioactive *in vitro* translation. C) CCL27 protein is detectable by sandwich ELISA in the supernatant of RD cells transfected with pCCL27, but not with the pVAX control (t=48hrs).

Figure 3. Immunization with SIV antigenic constructs elicits potent IFN- γ secretion and highly proliferative CD8⁺T cells

A) IFN- γ ELISpot was performed on lymphocytes isolated from peripheral blood of rhesus macaques 2 weeks post each immunization. Results are graphed as IFN- γ secreting cells/ 10^6 PBMCs in response to each antigen with values from media wells subtracted. Data are shown as averages for each group of macaques following each immunization. B,C) PBMCs from immunized macaques were stained with Carboxyfluorescein succinimidyl ester (CFDA-SE) and cultured for 5 days in the presence of media (negative control), SIV Gag, Env, or Pol peptides, or ConA (positive control). Cells were then stained for surface markers

(CD3, CD4, CD8) and run on flow cytometry. B) An example of proliferation in an immunized macaque. C) The average proliferation of CD8+ T cells in response to each SIV antigen from groups of macaques 4 weeks after each vaccination.

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Figure 4. Co-delivery of CCL27 does not enhance the magnitude of cytokine secretion in the periphery

PBMCs from immunized macaques were examined for cytokine secretion in response to stimulation with SIV peptide antigens (Gag, Pol or Env) in the presence of Golgi transport inhibitors and CD107a for 5 hours. Intracellular cytokine staining (ICS) was carried out from PBMCs following the final immunization. Data is graphed after values from media wells were subtracted. A) The total magnitude of cytokine secretion (and degranulation) was determined by adding the total number of cytokine secreting cells for each antigen for either CD4+(CD8-) or CD8+ (CD4-) T cells. The contribution of each function to the overall CD4+ and CD8+ T cell responses are shown in (B).

Figure 5. Co-delivery of CCL27 augments cytokine secretion in the lung

Levels of CD107a and IFN- γ expression by CD4+ T cells isolated from the periphery (PBMCs) or the mucosa (BAL) of immunized macaques were measured by ICS. Cells were stimulated with SIV Env for 5 hours in the presence of Golgi transport inhibitors. The percent of CD4% T cells responding minus background values in media only wells are shown.

Figure 6. DNA immunization elicits potent memory responses

Memory responses are robust in the periphery. PBMCs were isolated from immunized macaques 23 weeks following the 4th immunization and assayed for IFN- γ secretion by ELISpot (A, B) and CD8+T cell proliferation (C). The numbers of IFN- γ secreting cells are graphed for individual vaccinated macaques (A) or as averages for each group are shown in (B). The average CD8+T cell proliferation as determined by CFSE proliferation assays are shown in (C).

Figure 7. CCL27 augments antigen-specific IgA at mucosal, but not systemic sites

Antigen specific IgG or IgA antibody levels in various samples from immunized macaques were determined by endpoint titer ELISAs and B cell ELISpot. A) Sera samples were added to plates coated with SIVmac239 Env gp130 recombinant protein or BSA and the endpoint titers of IgG were determined by the O.D value in antigen specific wells that were two-fold over BSA values. Endpoint titers of antigen specific IgA, were also measured in the sera (B), bronchial lavage (C), and fecal pellets (D) by ELISAs. Mucosal samples were taken 10 weeks post the 4th immunization. Statistical differences are noted with a * ($p < 0.05$) and were determined by student's t-test.

Table 1
Study design and immunization groups

Groups of Chinese rhesus macaques (n=5) were immunized with RNA- and codon-optimized DNA constructs: SIV Gag, Pol, Env +/- rhesus CCL27 (1.0 mg each construct). A total of 4 immunizations were given intramuscularly with electroporation 6 weeks apart. The naïve group received saline and electroporation. 10 weeks later macaques were challenged given a high dose intrarectal challenge with SIVmac251.