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C3d Enhances Immune Responses Using Low Doses of DNA Expressing the HIV-1 Envelope from Codon-Optimized Gene Sequences

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Abstract

DNA vaccinations effectively induce both humoral and cellular immune responses to immunogens from diverse infectious agents. However, DNA vaccines expressing the HIV-1 envelope glycoprotein (Env) are poorly immunogenic when expressed from wild-type (wt) DNA sequences. Two recent approaches used to enhance the immunogenicity of Env expressed from a DNA vaccine are the fusion of the molecular adjuvant, C3d, to a soluble form of Env and the use of codon-optimized (co) *env* gene inserts. Independently, each approach enhances antibody titer and cellular responses against Env expressed from gene inserts. The goal of this study was to examine if both codon-optimization of *env* gene inserts and C3d conjugation to Env could function in a synergistic manner to enhance immunogenicity. Mice (BALB/c) were inoculated with decreasing doses (2.0 µg, 0.2 µg or 0.02 µg) of co DNA expressing Env alone or fused to three copies of murine C3d (mC3d₃) gene. Mice vaccinated with the highest dose (2.0 µg) of DNA had high anti-Env specific antibody titers regardless of the addition of mC3d₃. At lower doses (0.2 µg and 0.02 µg) of DNA, mice vaccinated with Env-mC3d₃ had enhanced immune responses compared to mice vaccinated with DNA expressing Env only. In addition, mice vaccinated with Env-mC3d₃ at the highest doses of DNA had enhanced interleukin-4 secreting cells, while mice vaccinated with the lowest dose of DNA had enhanced interferon-gamma secreting cells. Therefore, both codon-optimization of *env* sequences and C3d conjugation to Env appear to enhance anti-Env antibodies in an independent and additive manner.

Keywords

DNA vaccine; gene gun; codon optimization; C3d; molecular adjuvant

INTRODUCTION

DNA vaccines provide a promising alternative for new vaccination strategies due to their ability to induce protective immunity against infectious (*i.e.* viruses, bacteria, and parasites) and non-infectious (*i.e.* tumors) agents through the induction of both humoral and cellular immune responses [13,22,30]. The development of an effective DNA vaccine against HIV-1 has been challenging due to the poorly immunogenic nature of the envelope glycoprotein (Env) when expressed from wild-type DNA sequences. DNA vaccines expressing the gp120 subunit of HIV-1 Env elicit transient antibody titers, which are relatively poor at neutralizing viral

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infection [15,25,27,28]. The inability of DNA expressing gp120 to elicit high titer, cross-reactive antibodies may be due to a variety of factors, including the long period required for Env-specific antibody maturation [7].

Two recent approaches used to enhance the immunogenicity of Env expressed from a DNA vaccine are 1) the fusion of the molecular adjuvant, C3d, to a soluble form of Env [5,16,21,32,36] and 2) the use of codon-optimized (co) *env* gene inserts [5,11,18,21,37]. Independently, each approach enhances antibody titer and cellular responses against Env compared to DNA plasmid expressing wild-type (wt) *env* gene inserts only. The fusion of C3d to an antigen results in enhanced immunogenicity to the fused antigen [5,16,17,19,21,24,31,32,35,36]. Rodents inoculated with DNA plasmids expressing HIV-1 Env fused to multiple copies of human or murine C3d (mC3d) had enhanced anti-Env specific IgG titers and accelerated affinity maturation of antibody [16]. In addition, higher titers of neutralizing antibodies were elicited in rodents vaccinated with gp120-mC3d₃ compared to gp120 alone [16]. The precise mechanism of C3d enhancement is unclear; however both CR2-dependent and CR2-independent pathways play a role in C3d immune enhancement [19].

Codon-optimized DNA sequences expressing Env have increased levels of protein expression, which results in significant increases in antibody titer and cellular responses compared to DNA expressing wt sequences [2,8,21,37]. Recently, mice immunized with DNA plasmids expressing mC3d₃ fused to monomeric or trimeric forms of the HIV-1 envelope expressed from codon-optimized gene inserts elicited high titer anti-Env antibody [5,21]. Although, no differences were detected in the cell-mediated immune response in mice vaccinated with DNA expressing Env alone or conjugated to mC3d₃ from codon-optimized sequences [21]. However, significant increases in Env-specific interferon-gamma (IFN γ) secreting T cells were detected from isolated splenocytes of mice vaccinated with wild-type DNA sequences expressing Env-C3d₃, but not Env alone [21]. Therefore, the immune enhancement effects of C3d may be attenuated when C3d is conjugated to an antigen expressed from codon-optimized gene sequences. One potential reason for the lack of enhancement in the level of anti-Env specific antibodies in mice vaccinated with codon-optimized DNA expressing the Env-C3d fusion compared to Env alone may be due to the increased protein expression from codon-optimized DNA sequences. Therefore, the goal of this study was to examine if the combination of codon-optimization of *env* sequences and C3d conjugation to Env could function in a synergistic manner to enhance humoral and cell-mediated immune responses to HIV-1 Env using lower doses of DNA.

MATERIALS AND METHODS

Plasmid DNA

pTR600, a eukaryotic expression vector, has been described previously [31,32]. Briefly, the vector was constructed to contain the cytomegalovirus immediate-early promoter (CMV -IE) plus intron A (IA) for initiating transcription of eukaryotic inserts and the bovine growth hormone polyadenylation signal (BGH poly A) for termination of transcription. The vector contains the Col E1 origin of replication for prokaryotic replication and the kanamycin resistance gene (Kan^r) for selection in antibiotic media.

Plasmids were constructed to express a soluble form of HIV-1 gp120 using wild type or synthetic genes optimized for expression in mammalian cells [2,18,37] from the HIV-1 isolates, YU-2, JR-FL, ADA, and R2 [5,16,21,32]. Each DNA vaccine was designated for the gene insert that the plasmid expressed. For example, DNA plasmids expressing gp120_{YU-2} from wild type sequences were designated wt-gp120_{YU-2}.

The first 32 amino acids were deleted from the N-terminus of gp120 and replaced with a leader sequence from the trypsin plasminogen activator (tpA). In addition, mC3d₃ was fused to the C-terminus of gp120 molecule and designated wt-120-mC3d₃ or co-120-mC3d₃. Each plasmid was amplified in *Escherichia coli* strain-DH5 α , purified using anion-exchange resin columns (Qiagen, Valencia, CA, USA) and stored at -20°C in dH₂O. Plasmids were verified by appropriate restriction enzyme digestion and gel electrophoresis. Purity of DNA preparations was determined by optical density reading at 260 nm and 280 nm. Each DNA vaccine inoculation contained < 50 fg/ μ g of endotoxin per DNA inoculation.

***In vitro* Expression of DNA Vaccines**

In vitro expression of vaccine plasmids were performed as previously described [5]. Briefly, ELISA plates were coated with 1.0 % of the total supernatant from cells (3×10^5) transiently transfected with each DNA vaccine plasmid (2 μ g). Each plate was blocked with non-fat dry milk (5 %) in PBS containing Tween 20 (PBS-T) (0.05% solution for 1 h at 25°C). After extensive washed with PBS-T, polyclonal human HIV-infected patient antisera (HIV-Ig, 1:5000 dilution in PBS-T) was added. Once again, after extensively washing, horseradish peroxidase-conjugated goat anti-human antiserum (Bio-Rad, Hercules, CA, USA) was added (1:8000 dilution in PBS-T for 1 h at 25°C). Following thorough washing, antibody complexes were incubated (1 h at 25°C) with TMB substrate (Sigma, Saint Louis, MO, USA) and the optical density (O.D.) of each well was determined by spectrophotometry. The amount of protein in the supernatants was determined by comparison to known concentrations of purified recombinant gp120 (HIV-1_{BaL}).

Western Blot Analysis

Protein expression from each DNA vaccine was determined by western as described previously [5]. Supernatant (1.5%) were examined on a 7% polyacrylamide/SDS gel and the resolved proteins were transferred onto PVDF membrane (Sigma, Saint Louis, MO, USA). Each membrane was incubated with HIV-Ig (1:5000 dilution in PBS-T for 1 h at 25°C). Following extensive washing with PBS-T, human antibodies bound to the membrane were detected using horseradish peroxidase-conjugated goat anti-human antiserum (Bio-Rad, Hercules, CA, USA) (1:8000 dilution in PBS-T for 1 h at 25°C). Antibody complexes bound to the membrane were detected using enhanced chemiluminescence (Pierce Biotechnology, Inc., Rockford, IL, USA).

Animals and Immunizations

Six to 8 week old BALB/c mice (Harlan Sprague Dawley, Indianapolis, IN, USA) were used for inoculations. Mice, housed with free access to food and water, were cared for under USDA guidelines for laboratory animals. Mice were anesthetized with 0.03-0.04 ml of a mixture of 5 ml ketamine HCl (100 mg/ml) and 1ml xylazine (20 mg/ml). Gene gun immunizations were performed on shaved abdominal skin using the hand held gene delivery system (Bio-Rad, Hercules, CA, USA) as described previously [5,16,17,24]. Mice were immunized with two gene gun doses containing 1.0 μ g per 0.5 mg of approximately 1 μ m gold beads (Bio-Rad, Hercules, CA, USA) at a helium pressure setting of 400 psi. DNA was delivered as 2 gene gun doses at a total concentration of 1.0 μ g each, therefore when lower doses of DNA were administered an appropriate amount of vector DNA was added to maintain that equal amounts of DNA were given per inoculation.

ELISA for Detection of Anti-Env Antibodies

An endpoint ELISA was performed to assess the titers of anti-Env, as described [5]. Briefly, plates were coated with gp120 (25 ng) overnight at 4°C. Each plate was blocked with non-fat dry milk (5%) in PBS-T (1 h at 25°C) and then extensively washed with PBS-T. Serial dilutions of antisera from vaccinated mice were allowed to bind (1 h) to coated plates. Subsequently,

the primary antisera were detected by anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA, USA). Following thorough washing, antibody complexes were incubated (1 h at 25°C) with TMB substrate (Sigma, Saint Louis, MO, USA) and the optical density (O.D.) of each well was determined by spectrophotometry. Endpoint dilution titers from vaccinated mice that were two-fold higher than sera from mice vaccinated with vector only were considered positive.

ELISpot Assays

Splenocytes from immunized mice were collected (week 10) and re-suspended (5 million cells/ml) in RPMI-1640 supplemented with 10% FCS. Each well of a 96-well PVDF-coated plate (Millipore, Billerica, MA, USA) was coated with antibodies that detected the anti-mouse cytokines, IFN- γ (10 μ g/ml) or IL-4 (4 μ g/ml) (*mAb R4-6A2*, *mAb BVD4-1D11*, Pharmingen, San Diego, CA, USA). Plates were blocked with complete RPMI (2 h at RT) and then washed with PBS-T (3X). Splenocytes were added to each well (500,000 cells/well) and were stimulated (40 h at 37°C) with a pool of peptides (2 μ g per peptide). The C1 pool contained ten overlapping (15 amino acids/peptide) peptides representing a T-helper epitope in the first constant region of gp120_{MN} (amino acids 40-60) [9]. The V3 loop pool contained four peptides (15 amino acids/peptide) representing the V3 loop of the consensus Clade B, B-subtype, HIV-1_{MN} and HIV_{RF}, and one peptide (10 amino acids/peptide) representing the HIV-1_{IIIB} V3 loop (RGPGRAFVTI) [20,23]. Plates were washed with PBS-T (3X) and were incubated (2 h at RT) with biotinylated anti-mouse IFN- γ (2 μ g/ml) or anti-mouse IL-4 (2 μ g/ml) (*mAb XMGI.2*, *mAb BVD6-24G2*; Pharmingen, San Diego, CA, USA). Following thorough washing, plates were incubated with peroxidase-labeled avidin (Sigma, St. Louis, MO, USA) in PBS (1/1000 dilution). Wells were washed with PBS-T and spots were developed by the addition of the substrate 3,3-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO USA) in Tris-HCl, (50 mM, pH 7.5) containing hydrogen peroxide (0.015%). Spots were counted with the aid of a stereomicroscope.

Statistic Analysis

For statistical analysis, a student's T test was employed. The difference between DNA vaccines expressing Env fused to multiple copies of mC3d was compared to DNA expressing Env alone for wt and each dose of co DNA. Differences were considered statistically significant when p-value was less than 0.05.

RESULTS

Expression of Vaccine Plasmids

Human 293T cells were transfected with various doses of DNA expressing gp120 $\pm$ mC3d₃ from codon-optimized sequences (2.0 μ g, 0.2 μ g, 0.02 μ g). Also, cultures of additional cells were transfected with only the highest dose (2.0 μ g) of gp120 $\pm$ mC3d₃ from wild type sequences. Each plasmid efficiently expressed its gene insert, however, cells transfected with co-gp120-DNA secreted 7-10 times more protein than cells transfected with wt-gp120-DNA (Fig. 1B). Similar results were observed in cells transfected with any of the co- or wt-gp120-mC3d₃-DNA, regardless of the dose of plasmid used for transfection (Fig. 1B). Cells transfected with co-gp120-DNA had the highest level of protein expression ($\sim$ 10 μ g/ml). As expected, there was a dose-dependent decrease in protein expression, which correlated with lower doses of transfected plasmid DNA (Fig. 1A & B). Interestingly, the expression of each co- or wt-gp120-DNA at each individual dose was higher than their gp120-mC3d₃-DNA counterpart (Fig. 1B). These results were verified in mouse fibroblast cells (NIH 3T3), however, while the pattern of expression was similar for each vaccine plasmid at each dose, $\sim$ 20-fold less protein was produced compared to 293T cells (data not shown).

Anti-Env Antibody Responses.

Mice (BALB/c) were vaccinated by gene gun (g.g.) with one of three doses (2.0 μ g, 0.2 μ g and 0.02 μ g) of co-gp120-DNA or co-gp120-mC3d₃-DNA or with a single dose (2.0 μ g) of wt-gp120-DNA or wt-gp120-mC3d₃-DNA (Fig. 2). As previously reported [16,31,32], mice vaccinated with wt-gp120-mC3d₃-DNA elicited significantly higher titers of anti-Env specific antibody compared to mice vaccinated with wt-gp120-DNA ($p > 0.05$) (Fig. 2A). However, both co-gp120-DNA and co-gp120-mC3d₃-DNA elicited similar titers of anti-Env antibody ($> 1:50000$) that was ~ 10 fold higher than antibody elicited by the corresponding wt-gp120-mC3d₃-DNA (Fig. 2A). Similar results were observed for all four gp120 proteins. Interestingly, at the highest dose (2.0 μ g), C3d did not enhance the anti-Env antibody titers when conjugated to gp120 expressed from codon-optimized sequences (Fig. 2B). However, the adjuvant effect induced by C3d was observed using lower doses of plasmid for vaccination. Mice vaccinated with 10-fold less co-gp120-mC3d₃-DNA (0.2 μ g) showed a slight enhancement in anti-Env specific antibody compared to co-gp120-DNA (Fig. 2). In addition, mice vaccinated with 100-fold less of co-gp120-mC3d₃-DNA (0.02 μ g) elicited 4-10 higher titers of anti-Env antibody compared to co-gp120-DNA and these titers resembled the anti-Env titers elicited by gp120-mC3d₃ expressed from wild type sequences (2.0 μ g).

Regardless of mC3d₃ conjugation to gp120, mice vaccinated with the highest doses (2.0 μ g or 0.2 μ g) of co-gp120 $\pm$ mC3d₃ had a steady rise in anti-Env antibodies ($1:10^4$ - $1:10^5$ at week 10) (Fig. 2B). As represented by vaccines expressing gp120_{YU-2} (Fig. 2B), mice vaccinated with co-gp120-DNA elicited slightly higher titers of anti-Env antibodies throughout the study compared to mice vaccinated with co-gp120-mC3d₃-DNA. The anti-Env titer was detected after the first inoculation and then rose sharply following the second and third vaccinations ($> 1:10^4$) at week 10) (Fig. 2B). In contrast, the lowest dose of co-gp120-DNA or co-gp120-mC3d₃ (0.02 μ g) elicited a slower rise in anti-Env antibodies (10^2 - 10^3 at week 10) (Fig. 2A). Interestingly, mice vaccinated with a 2.0 μ g dose of wt-gp120-mC3d₃-DNA elicited similar titers of anti-Env antibody as mice vaccinated with the lowest dose (0.02 μ g) of co-gp120-mC3d₃-DNA (Fig. 2B). Similar results were observed using any of the other three envelopes (data not shown).

Cell-Mediated Immune Responses Elicited by Env-mC3d₃

Splenocytes isolated from mice vaccinated with wt-gp120-mC3d₃-DNA elicited a higher number of cytokine secreting cells compared to mice vaccinated with wt-gp120-DNA (Table 1). For splenocytes stimulated with the V3 loop peptides, the number of cytokine secreting cells was statistically higher in mice vaccinated with co-gp120-DNA (2.0 μ g) than mice vaccinated with wt-gp120-DNA ($p < 0.05$, for IFN γ or IL-4). However, there was no statistical difference between the number of IFN γ secreting cells elicited in wt-gp120-mC3d₃-DNA vaccinated mice and co-gp120-mC3d₃-DNA vaccinated mice (Table 1). In addition, there was little to no statistical difference between the number of cytokine secreting cells in mice vaccinated with co-gp120-DNA and co-gp120-mC3d₃-DNA, regardless of the dose used for vaccination (Table 1).

In comparison, splenocytes stimulated with the C1 pool of peptides, from mice vaccinated with co-gp120-mC3d₃-DNA elicited statistically higher numbers of IL-4 secreting cells than splenocytes from wt-gp120-mC3d₃-DNA vaccinated mice ($p < 0.05$). However, mice vaccinated with co-gp120-mC3d₃-DNA had higher numbers of mIL-4 secreting splenocytes compared to co-gp120-DNA vaccinated mice following stimulation with the C1 pool of peptides ($p < 0.05$). In contrast, the number of INF- γ secreting cells in mice vaccinated with the lowest dose of co-gp120-mC3d₃-DNA was higher than co-gp120-DNA vaccinated mice. Lastly, there was a dose dependent increase in the number INF- γ secreting cells in mice

vaccinated with co-gp120, but little evidence for a dose dependency in IL-4 secretion (Table 1).

DISCUSSION

Despite a variety of vaccine approaches that elicit diverse immune responses, currently no HIV/AIDS vaccine is effective at preventing viral infection [1,6,29,40]. A lack of understanding of the immune correlates of protection is a primary reason for the ineffectiveness of past vaccines. However, the elicitation of humoral, cell-mediated, and innate immune responses in both the systemic and mucosal compartments appears necessary for an effective vaccine for HIV-1 infection [1,29,40].

DNA vaccines induce all three of these immune responses in both parental and mucosal immunization strategies and therefore, have provided an alternative approach for HIV-1 vaccine development compared to live-attenuated or subunit/protein immunizations [22,30]. However, the effective use of DNA vaccines in small rodent models has not translated into the elicitation of high titer, protective immunity in larger sized mammals, such as monkeys and humans [1,29]. Priming the immune system with a DNA vaccine(s) followed by one of a variety of booster inoculations (i.e. viral vectors or recombinant proteins) is an effective strategy for eliciting high titer immune responses to HIV-1 antigens [1,29].

In order to enhance the immune responses elicited by a DNA vaccine, we used two effective approaches which, independently, enhance antibody titer and cellular responses against HIV-1 envelope glycoproteins; the use of C3d as a molecular adjuvant [5,16,19,21,24,31,32] and enhanced protein expression from codon-optimized gene sequences [2,3,10,37,41]. Therefore, in this study, DNA vaccines expressing one of four gp120 molecules from wild type HIV-1 gene sequences or sequences that were optimized for expression in mammalian cells (codon-optimized) were used to immunize mice. Each gp120 gene was fused with the three tandem copies of the gene that encodes for the murine C3d. The goal of this study was to determine if the adjuvant effects produced by C3d could be combined with codon-optimization in order to increase the level of immunity elicited from a DNA vaccine.

The level of protein expression from wild-type gene sequences is 10-100 fold lower than sequences that have been optimized from expression in mammalian cells [2,10,14,37,41]. Cells transfected with plasmids expressing gp120 or gp120-mC3d₃ from codon-optimized sequences secreted 7-10 fold higher amounts of protein than the corresponding wild-type sequences (Fig. 1), which were independent of the envelope isolate used as an antigen. This increased expression of Env from codon-optimized gene inserts directly correlates to increased immunogenicity in mice, compared to the poor immunogenic nature of *env* expressed from wild-type gene inserts when vaccinated with the same 2.0 µg dose of DNA (Fig. 2) [2,37]. When the dose of codon-optimized DNA is reduced by 10 and 100-fold, the titer of anti-Env specific antibodies elicited in vaccinated mice is progressively decreased. The inoculation of increasing doses of codon-optimized *gag* shows a similar relationship between the level of expression of the antigen and the titer of antibody elicited [41].

The molecular adjuvant, C3d, has been shown to enhance the immunogenicity of viral, bacterial, and self antigens (T-dependent and T-independent) when inoculated as either DNA or protein in both inbred and outbred mice strains [4,5,12,16,17,19,21,24,31,32,35,36,38,39]. Overall, the inoculation of C3d fused antigens as protein immunogens seems to produce more consistent results than DNA expressing the same protein *in-vivo*. For example, the fusion of C3d to the human chorionic gonadotropin (hCGβ) provided a robust enhancement in anti-hCGβ specific antibody titers in mice when administered as recombinant protein [39], but inhibited antibody production when expressed from a DNA vaccine [34]. Although, one possible reason for these inconsistencies may be in the methodologies used for the DNA

vaccination of hCG β , which were different than previous C3d adjuvant studies [4,5,16,17,19, 21,24,31,32,36]. It is unclear if the use of multiple adjuvants or technical manipulations interfered with the ability of C3d to elicit an effective immune response [34], however, this same antigen inoculated as a protein vaccine elicited a robust enhancement in antibody titer [39].

Enhanced immunogenicity by C3d correlates with the number of C3d molecules fused to the antigens. The addition of one copy of C3d to an antigen appears to inhibit antibody secretion [12,16], however, multimers (>2 copies) of C3d elicit enhanced immune responses. Therefore, it is unclear why the fusion of 2 copies of C3d to the bovine rotavirus VP7 and bovine herpesvirus type 1 glycoprotein D resulted in the inhibition of antibody responses [33]. However, since the fusion of C3d enhances the immune response to a variety of different viral envelope proteins, the origin of the protein may be important [4,5,16,17,24,31,38]. The variability in C3d enhanced immune responses may reflect the type of antigen, the form of the antigen, or the dose of the antigen administered.

Similar to previous reports from our laboratory [4,5,16,32,36], C3d effectively enhanced the level of anti-Env antibodies elicited in mice vaccinated with wild-type gp120-mC3d₃ (2.0 μ g), compared to wild-type gp120 (2.0 μ g) from any of the four HIV-1 isolates (Fig. 2). Using the same dose of DNA (2.0 μ g) these enhancement effects by C3d were masked in vaccines expressing gp120-mC3d₃ from codon-optimization of *env* gene inserts (Fig. 2). Compared to DNA expressing gp120 alone, the enhancement in anti-Env responses in mice receiving gp120-mC3d₃ was progressively lost as the dose of codon-optimized DNA was increased (Fig. 2). In contrast, mice vaccinated with 10- (0.2 μ g) or 100-fold (0.02 μ g) less codon-optimized gp120-mC3d₃-DNA elicited enhanced anti-Env antibodies compared to the same dose of codon-optimized gp120-DNA (Fig. 2). Interestingly, mice vaccinated with 0.02 μ g of codon-optimized gp120-mC3d₃-DNA elicited the same pattern of immune responses as observed in mice vaccinated with 2.0 μ g wild-type gp120-mC3d₃-DNA (Fig. 2). This result was not surprising since the level of expression was similar between these two groups (Fig. 1). The titer of anti-Env antibody elicited appeared to correlate to protein expression. Overall, there was a clear relationship between the amount of antigen expressed *in vitro* to the titer of antibody elicited; when comparing the level of expression from each dose of DNA (Fig. 1) to the titer of antibody elicited (Fig. 2). In addition, the increased protein expression at the highest dose of codon-optimized DNA, compared to wild-type DNA, is most likely responsible for the masking of C3d enhancement in antibody titer, as previously reported [5,21].

The inoculation of DNA expressing viral antigens elicits potent cell-mediated immune responses [22,30]. In the present study, cell-mediated immune responses were determined by ELISpot analysis using previously described peptides specific for MHC class I or class II [9, 20,23]. There was a direct relationship between the number of IL-4 and IFN- γ secreting cells and the amount of antigen expressed (Table 1). Splenocytes from mice vaccinated with higher doses of codon-optimized DNA, which express higher amounts of protein, had increased numbers of cytokine secreting cells (Table 1). Similar dose dependent increases in cytokine secreting cells have been reported in mice inoculated with codon-optimized DNA expressing *gag* sequences [41].

In addition to enhancing the titers of anti-Env specific antibodies, C3d increases the magnitude of IL-4 and IFN- γ specific secreting splenocytes to various antigens [21,24,26,39]. Inoculation by gene gun of DNA expressing the highly immunogenic hemagglutinin (HA) protein from influenza virus displays T helper (Th) type 2 (Th₂) biased response, regardless if splenocytes were stimulated with MHC class I or class II peptides [24,26]. These results are consistent with the notion that intradermal inoculation via gene gun of plasmid DNA expressing secreted antigens elicits a Th₂ response [26]. The fusion of C3d to HA, enhanced the magnitude of the

response toward a Th₂ bias, however, C3d does not appear to alter the immune bias already established by the antigen. In addition, mice vaccinated with wild-type DNA expressing gp120-mC3d₃ had an increase in IFN- γ specific responses compared to mice vaccinated with wild-type DNA expressing gp120 alone [21], when splenocytes were stimulated with a MHC class I peptides. C3d fusion to an antigen increases the magnitude of the already established cell-mediated immune response, whether it is a T helper type 1 (Th₁) or a Th₂ response.

Inoculation of wild-type gp120-DNA resulted in the secretion of low levels of IL-4 and IFN- γ when stimulated with either MHC class I or class II peptides (Table 1). Splenocytes from mice vaccinated with wild-type gp120-mC3d₃-DNA stimulated with peptides specific for either MHC class I or class II had significantly increased numbers of both IL-4 and IFN- γ producing cells compared to gp120 alone (Table 1). In contrast, regardless of the dose of codon-optimized DNA, stimulation of splenocytes with peptides specific for MHC class I resulted in a mixed T helper response; production of equal numbers of IL-4 and IFN- γ secreting cells (Table 1). However, stimulation of sister cultures of splenocytes with peptides specific for MHC class II resulted in a clear bias toward a Th₂ response, regardless of the dose of DNA inoculated. The fusion of C3d to such antigens does not appear to alter the responses elicited by these antigens, but acts to increase the magnitude of the established response.

Overall, a similar pattern of elicited humoral and cell-mediated immune responses correlates with the doses of codon optimized DNA administered to the mice. These effects appear linked to the overall expression of protein from these codon-optimized gene inserts. Higher doses of codon-optimized DNA secreted more envelope and correlated with higher titers of anti-Env antibody, as well as increased the numbers of cytokine secreting cells, compared to these same codon-optimized DNA vaccines administered at lower doses or expressed from wild-type gene inserts.

Together, these data indicate the fusion of C3d and codon-optimization of gene sequences function both independently and synergistically, however this effect is dependent on the dose of DNA plasmid administered *in vivo*. Since the potency of DNA vaccines in rodents has not translated into larger mammals, there is a need for a molecular adjuvant, such as C3d, which can act independently and enhance the effects of codon optimization. We have previously reported that the human homologue of C3d enhances immunity as effectively as the mouse homologue [16]. Therefore, future studies should be aimed at validating the use of C3d in higher primates. One potential benefit of using C3d in a DNA vaccine is the ability to enhance immune responses using lower doses of DNA. In addition, the conjugation of C3d to a viral antigen is capable of improving the level of neutralizing antibodies [5,21] and providing protection from a viral challenge [24] compared to the antigen alone. The findings of this study show that improvements in both humoral and cell-mediated immune responses can be achieved both by the fusion of the molecular adjuvant, C3d, to HIV-1 Env alone or in combination with codon-optimization.

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Fig. (1).

Expression of wild-type and codon-optimized gp120 $\pm$ mC3d₃. A) Representative western blot of vaccine plasmids expressing HIV-1_{YU-2} envelopes. Supernatants from transiently transfected 293T cells were visualized using 7% SDS-PAGE. B) The level of *in vitro* expression was determined relative to lane 4 (gp120, 2.0 μ g co DNA), which had the highest level of expression. Lane 1: Molecular weight marker; lane 2: wt gp120; lane 3: wt gp120mC3d₃; lane 4: co gp120 (2.0 μ g); lane 5: co gp120mC3d₃ (2.0 μ g); lane 6: co gp120 (0.2 μ g); lane 7: co gp120mC3d₃ (0.2 μ g); lane 8: co gp120 (0.02 μ g); lane 9: co gp120mC3d₃ (0.02 μ g). All isolates tested displayed similar levels and patterns of expression at each dose of co DNA.

Fig. (2).

Endpoint dilution titer for each HIV-1 isolate. Endpoint dilution titers of week 10 sera for each of the four HIV-1 isolates (YU-2, ADA, JR-FL, or R2). BALB/c mice (n=5 per group) were primed at day 0 with each vaccine construct using 2 μ g of total DNA inoculated *via* gene gun, and boosted at weeks 4 and 8 with an additional 2 μ g of DNA *via* gene gun. Sera were collected prior to and two weeks after each inoculation and was assayed for specific IgG levels by ELISA. Ninety-six well plates were coated with 25 ng of gp120_{YU-2} recombinant protein. Data are represented as the average of five mice. Preimmune sera from mice had no detectable specific IgG. Endpoint dilution titers were conducted by diluting the sera until O.D. values reached background. B) The temporal pattern of anti-Env immune responses is shown. Sera were collected prior to and two weeks after each inoculation and was assayed for specific IgG levels by ELISA.

Table 1.
ELISpot Analysis was Performed on Splenocytes from Vaccinated Mice

	IL-4 ^a	IFN-γ ^a
V3 loop pool		
co-gp120 (2.0 μg)	14.0 ± 4.4 #	12.7 ± 4.9 #
co-gp120-mC3d ₃ (2.0 μg)	31.2 ± 17.5	19.3 ± 1.7 #
co-gp120 (0.2 μg)	9.0 ± 5.1	6.0 ± 3.6
co-gp120-mC3d ₃ (0.2 μg)	15.4 ± 4.9	12.7 ± 7.6
co-gp120 (0.02 μg)	9.8 ± 5.2	0.0 ± 0.0
co-gp120-mC3d ₃ (0.02 μg)	13.4 ± 5.6	12.7 ± 2.5 *
wt-gp120 (2.0 μg)	2.0 ± 1.2	0.0 ± 0.0
wt-gp120-mC3d ₃ (2.0 μg)	25.3 ± 6.0 *	14.0 ± 2.0 *
C1 pool		
co-gp120 (2.0 μg)	66.4 ± 12.6 #	28.2 ± 5.9 #
co-gp120-mC3d ₃ (2.0 μg)	114.0 ± 15.3 *#	41.3 ± 12.1
co-gp120 (0.2 μg)	44.0 ± 11.8	20.7 ± 7.3
co-gp120-mC3d ₃ (0.2 μg)	117.2 ± 28.3 *#	25.3 ± 7.7
co-gp120 (0.02 μg)	58.4 ± 14.3 #	8.1 ± 2.8
co-gp120-mC3d ₃ (0.02 μg)	127.0 ± 32.4 #	18.7 ± 1.4 *
wt-gp120 (2.0 μg)	22.2 ± 4.2	5.6 ± 2.9
wt-gp120-mC3d ₃ (2.0 μg)	42.8 ± 8.2 *	22.4 ± 2.5 *

^a IFN-γ and IL-4 specific secreting cells were measured by re-stimulating splenocytes with peptides from 2 separate pools, C1 pool and V3 loop pool.
wt=wild type, co=codon optimization

* Significant difference (p<0.05) between gp120 and gp120-mC3d₃

Significant difference (p<0.05) between co-(at each dose) and wt-gp120 ± mC3d₃