Robust antibody and cellular responses induced by DNA-only vaccination for HIV

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Background: HVTN 098, a randomized, double-blind, placebo-controlled trial, evaluated the safety, tolerability and immunogenicity of PENNVAX®-GP HIV DNA vaccine, administered with or without plasmid IL-12 (pIL-12), via intradermal (ID) or intramuscular (IM) electroporation (EP) in healthy, HIV-uninfected adults. The study tested whether PENNVAX®-GP delivered via ID/EP at 1/5th the dose could elicit equivalent immune responses to delivery via IM/EP, and if inclusion of pIL-12 provided additional benefit.

Methods: Participants received DNA encoding HIV-1 env/gag/pol in three groups: 1.6mg ID (ID no IL-12 group, n=20), 1.6mg ID + 0.4mg pIL-12 (ID+IL-12 group, n=30), 8mg IM + 1mg pIL-12 (IM+IL-12 group, n=30) or placebo (n=9) via EP at 0, 1, 3 and 6 months. Results of cellular and humoral immunogenicity assessments are reported.

Results: Following vaccination, the frequency of responders (response rate) to any HIV protein based on CD4+ T-cells expressing IFN-γ and/or IL-2 was 96% for both the ID+IL-12 and IM+IL-12 groups; CD8+ T-cell response rates were 64% and 44%, respectively. For ID delivery, the inclusion of pIL-12 increased CD4+ T-cell response rate from 56% to 96%. The frequency of responders was similar (>90%) for IgG binding Ab to gp140 consensus […]

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**Abstract**

**Background:** HVTN 098, a randomized, double-blind, placebo-controlled trial, evaluated the safety, tolerability and immunogenicity of PENNVAX®-GP HIV DNA vaccine, administered with or without plasmid IL-12 (pIL-12), via intradermal (ID) or intramuscular (IM) electroporation (EP) in healthy, HIV-uninfected adults. The study tested whether PENNVAX®-GP delivered via ID/EP at 1/5th the dose could elicit equivalent immune responses to delivery via IM/EP, and if inclusion of pIL-12 provided additional benefit.

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**Conclusion:** PENNVAX®-GP DNA induced robust cellular and humoral immune responses, demonstrating that immunogenicity of DNA vaccines can be enhanced by EP route and inclusion of pIL-12. ID/EP was dose-sparing, inducing equivalent, or in some aspects superior, immune responses compared to IM/EP.

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Introduction

DNA has been included as part of the vaccine regime for several candidate HIV vaccines contributing to robust immunogenicity (e.g., (1-3)). Although the immune response due to the DNA component was initially relatively poor, this has increased over time largely due to plasmid optimization and improved DNA formulation (4). Perhaps the most marked increase in immunogenicity has resulted from delivery via in vivo electroporation (EP). Although there were initial concerns about the feasibility and tolerability of EP vaccination in vivo, several trials have shown acceptability of EP among study participants and enhanced immune responses when delivered by the intramuscular (IM) (5-8) and intradermal (ID) (9, 10) routes. The HIV Vaccine Trials Network (HVTN) 098 study, reported here, explored improvements in plasmid and construct design, formulation, as well as enhanced delivery in healthy, HIV-1-uninfected study participants in a multicenter clinical trial.

Previously, the HVTN evaluated a multigene HIV-1 DNA immunogen encoding HIV-1 clade B gag, pol and env plasmids administered with plasmid IL-12 (pIL-12) by IM injection followed by EP (6). This early generation adaptive IM/EP markedly increased both the CD4+ and CD8+ T-cell responses when compared to a similar vaccine regimen in a prior study administered without EP (6). After the third vaccination, the CD4+ T-cell and CD8+ T-cell response rates increased from 19% to 81% and 7% to 52%, respectively.

In vivo EP was developed to improve the immunogenicity of DNA for therapeutic applications. The first DNA vaccine trial to demonstrate clinical efficacy included delivery via CELLECTRA® IM/EP (8) using consensus synthetic DNA cassettes (encoding HPV 16/18 E6 and E7 proteins) for therapy of an HPV-associated cervical intraepithelial neoplasia (CIN). A significant difference in regression of CIN was noted among vaccine recipients compared to placebos, and complete clearance of virus was associated with CD8+ T cells trafficking to the cervical tissue.
Skin is an attractive target tissue for delivering DNA vaccines for multiple reasons: 1) skin is the largest organ of the human body and readily accessible; 2) it harbors Langerhans and other antigen-presenting cells, so it is capable of developing a broad immune response to antigens; and 3) is less invasive and avoids stimulation of muscle tissue (11, 12). Although delivery via IM/EP has been well tolerated, EP with intradermal (ID) administration (ID/EP) may improve tolerability as the electrodes do not penetrate as deeply. A recent clinical study demonstrated both cellular and humoral immunogenicity for DNA vaccine targeting the Ebola virus glycoprotein that was administered via ID/EP (10).

Preclinical studies have demonstrated that the immunogenicity of DNA vaccines can be substantially increased by the use of cytokine adjuvants, such as IL-12 (13-15), although there are limited data demonstrating enhanced immunogenicity in clinical studies. The HVTN 087 study tested HIV-1 multiantigen DNA with increasing doses of pIL-12 administered via an IM/EP approach, followed by recombinant vesicular stomatitis virus (VSV) expressing HIV-1 Gag (16). High-dose pIL-12 increased the magnitude of CD8+ T-cell responses after the VSV boost compared to no pIL-12. In contrast, CD4+ T-cell responses following the DNA prime were higher in the group that did not receive the pIL-12. Two other HVTN studies tested DNA plus pIL-12 with EP (HVTN 080) and without EP (HVTN 070) (6). In HVTN 070, the addition of pIL-12 in the absence of EP did not provide any benefit. In HVTN 080, however, a study that utilized EP, CD4+ T-cell response rates increased from 44% to 81% after the third vaccination when pIL-12 was included, although this was not statistically different likely due to the small sample size for the group without pIL-12. CD8+ T-cell responses were also higher in the group with pIL-12 (52% compared to 33%). Responses were most often detected to Gag or Pol with few responses to Env.

The HVTN 098 trial, reported here, was designed to build on the promising induction of CD4+ and CD8+ T-cell immune responses demonstrated in HVTN 080 (6). Constructs were further designed with the following important enhancements. We focused on increasing the
magnitude and breadth of Env-specific immune responses in HVTN 098 by designing and including two env plasmids encoding both RNA- and codon-optimized clade A and C consensus Env immunogens (rather than the single env clade B plasmid in HVTN 080). The env plasmids included a substitution of an optimized IgE leader as well as the deletion of their cytoplasmic tail improving surface expression. This change was added to further improve B cell engagement while maintaining T cell response induction. We also increased the dose of the HIV-1 env A and env C plasmids relative to the gag and pol plasmids. The gag and pol plasmids were modified to have an improved design as they encoded multi-clade consensus immunogens vs. the gag and pol plasmids that encoded a primary strain clade B immunogen in HVTN 080. In addition to obtaining safety and tolerability data, HVTN 098 was designed to address whether administration via ID/EP CELLECTRA® is equivalent to IM/EP though a fraction of the antigen dose is utilized, as well as whether inclusion of an IL-12 plasmid as a genetic adjuvant provided additional benefit for induction of cellular and humoral responses.
Results

Safety and tolerability

A total of 94 participants were enrolled; nine were placebo and 85 were vaccine recipients (Fig. 1). Details on the safety, tolerability and acceptability of these vaccinations by either the ID or IM route with EP are reported separately (Edupuganti et al, submitted). Overall, vaccinations were safe and well-tolerated. IM/EP was associated with more pain as compared to ID/EP. Most participants in the ID/EP group had skin lesions including scars and hypo- or hyperpigmentation, and these resolved within 6 months in half of these participants. The majority (82%) of both IM/EP and ID/EP participants reported that the level of discomfort was acceptable, and all but two said they would be willing to undergo EP vaccination for a serious disease.

T-cell responses

CD4+ and CD8+ T cells expressing IFN-γ and/or IL-2 were detected post-immunization following ex vivo stimulation with any HIV-1 Env, Gag and Pol peptide pool (Fig. 2A and Fig. S1). CD4+ T-cell response rates after the fourth ID and IM immunization were highest in the groups receiving pIL-12 (96%), and significantly lower (p=0.002, q=0.04) for the ID no IL-12 group (56%) compared to the ID+IL-12 group (96%). However, the response magnitudes were not different between the ID groups after either the third or fourth vaccinations. Comparing the IM + IL-12 group vs. the ID + IL-12 group, the response magnitude after the third vaccination was higher for the IM group (medians of 0.14% of CD4+ T cells for ID + IL-12 vs. 0.26% for IM + IL-12, p=0.006, q=0.07), but the magnitudes of the two groups were not significantly different two weeks after the fourth vaccination (0.19% vs. 0.23%). The fourth vaccination did not significantly increase the CD4+ T-cell response rate to any HIV antigen over the response to the third vaccination; however, the response magnitude after the fourth vaccination was significantly
increased in the ID + IL-12 group as compared to after the third vaccination (from median of 0.14% to 0.19%, p=0.004, q=0.03).

HIV-specific CD8+ T-cell response rates were similar across treatment groups, with the highest response rates in the ID + IL-12 group (64% as compared to 56% for ID no IL-12 and 44% for IM + IL-12; p \geq 0.18, q \geq 0.7) after the fourth immunization. The response magnitudes were generally higher for CD8+ T cells as compared to CD4+ T cells (medians ranging from 0.33 to 0.43% for CD8+ as compared to 0.19 to 0.23% for CD4+ T cells after the fourth vaccination) but were not significantly different between the groups. The fourth vaccination boosted the magnitude of the CD8+ T-cell responses in all three groups (p \leq 0.05, q<0.14).

At six months after the fourth vaccination, response rates remained high for both CD4+ and CD8+ T cells, with a significant decrease only for CD4+ T cells in the ID + IL-12 group (from 96% to 66%, p=0.004, q=0.03). Remarkably, the CD8+ T-cell response rates remained similar at six months. Response magnitudes for CD4+ and CD8+ T cells were significantly reduced at the later timepoint for both of the ID groups (q<0.06), but not for the IM group.

The immunogenicity of the env, gag and pol components of the vaccine differed. CD4+ T-cell response rates were highest for Env (50, 86 and 93% for the ID no IL-12, ID + IL-12 and IM + IL-12 groups, respectively), followed by Gag (31, 50 and 56% for the ID no IL-12, ID + IL-12 and IM + IL-12 groups, respectively), and nearly absent for Pol (Table 1). CD8+ T-cell responses were primarily induced in response to Env (50, 57 and 44% for the ID no IL-12, ID + IL-12 and IM + IL-12 groups, respectively), with only a few responding individuals for Gag and Pol.

Polyfunctionality analysis using COMPASS demonstrates a highly diverse functional profile among the responding CD4+ T cells to Env (Fig. 2B and C). Among the seven functional markers examined, many cells expressed combinations including up to five of these markers. Only two of the markers, IL-4 and IL-17 were not detected among the responding cells. IFN-\gamma,
IL-2, TNF-α and CD40L were the functions most commonly detected, and granzyme B was co-expressed in some cases. There was a less diverse functional profile for the responding CD8+ T cells as compared to CD4+ T cells, including cells producing IFN-γ, IL-2, TNF-α and granzyme B or subsets of these markers. The distribution of functional profiles was similar for all treatment groups. The Env-specific CD4+ T-cell polyfunctionality score derived from COMPASS was higher for the ID + IL-12 group compared to the ID no IL-12 group at two weeks after the third (p=0.04, q=0.12) and fourth vaccinations (p=0.02, q=0.10). The CD8+ T-cell polyfunctionality score was not significantly different between treatment groups for Env, but for Gag, it was higher for the ID + IL-12 group compared to the IM + IL-12 group at two weeks after the fourth vaccination (p=0.006, q=0.06, data not shown), although the magnitudes of the scores are much lower than for Env. In summary, the cellular immunogenicity revealed similar response rates and magnitudes for both the ID and IM routes (with pIL-12) after the fourth vaccination. At six months after the fourth vaccination, these responses were maintained for CD8+ T cells and only modestly decreased for CD4+ T cells. There was a benefit of including pIL-12 with the ID delivery as reflected in increased CD4+ T-cell response rate and increased CD4+ T-cell polyfunctionality.

Antibody Responses

Serum IgG antibodies binding Env gp140, gp120, gp41, and Gag p24 proteins were induced in all treatment groups (Fig. 3). Antibody response rates to consensus gp140 were uniformly high and similar for all treatment groups (up to 100%) after the fourth vaccination, but the response magnitude was higher for the ID + IL-12 group vs. the IM + IL-12 group (median MFIs of 9690 vs. 1967, p=0.008, q=0.08). Response rates and magnitudes were markedly lower for gp120 compared to gp140, with response rates up to 100% for gp140 and only up to 57% for gp120. This difference is likely due to additional epitope specificities (e.g.,
conformational) present in the gp140 antigens and not in the gp120 antigens. The gp120 response rates were not different between the groups, but response magnitude was higher for the ID + IL-12 group compared to IM + IL-12 two weeks after the fourth vaccination (p=0.03, q=0.10). For gp41, IgG response rates were high for both ID groups compared to the IM + IL-12 group; the IM + IL-12 response rate was significantly lower than that for the ID + IL-12 group after the third vaccination (p<0.0001, q=0.001) and also after the fourth vaccination (p=0.007, q=0.08) although response magnitudes were not different.

There was a boosting effect of the fourth dose, as reflected in significantly higher magnitudes between the third and the fourth vaccinations for gp140 in the ID no IL-12 group (p=0.006, q=0.02) and the IM group (p=0.03, q=0.05), as well as for gp41 in both ID groups (p≤0.04, q≤0.06) and for gp120 in all three groups (p≤0.04, q≤0.06). Response rates six months after the fourth vaccination remained high for gp140, but were significantly reduced for gp41 and gp120 among nearly all the treatment groups (p from 0.13 to 0.001, q from 0.20 to 0.02), suggesting that the most durable antibody responses were to conformational epitopes present in gp140 Env antigens and not in gp120 and gp41. The magnitudes of responses were significantly reduced at six months after the fourth vaccination for all three Env antigens in all three groups (p≤0.02, q≤0.02). Response rates for Gag p24 were lower than for the Env antigens, with a maximum of 57% in the ID + IL-12 group after the fourth vaccination. Response rates and magnitudes were not significantly different between groups.

Response rates and magnitudes for gp140 Env proteins representing clades A, B and C were high and similar to the consensus gp140 responses, even though there was no clade B-specific immunogen administered as part of the vaccine (Fig. S2). Similar to the consensus, there were no significant differences in response rates between treatment groups. Response magnitudes were lower for the IM + IL-12 group at all three timepoints as compared to the ID + IL-12 group, and the differences were significant for clade B for all these timepoints (p≤0.03,
The fourth vaccination boosted the magnitude of the response for all treatment groups against clades A and B, and for the ID no IL-12 group against clade C ($p \leq 0.04$, $q \leq 0.06$).

Response rates against these gp140 antigens remained high at six months after the fourth vaccination, with significant drop in the response rate only in the ID + IL-12 group for clade A ($p=0.02$, $q=0.07$) and clade C ($p=0.03$, $q=0.1$). Response magnitudes at this later timepoint were significantly reduced for all treatment groups and all gp140 antigens ($p \leq 0.002$, $q \leq 0.002$).

Response magnitude after the third vaccination was higher for the ID + IL-12 group vs. the ID no IL-12 group for all three antigens ($p \leq 0.04$, $q \leq 0.3$), showing a potential benefit of IL-12 in enhancing the antibody response to near maximal with only three doses of vaccine.

To examine IgG anti-Envelope binding antibody breadth to gp120 and specifically to clade C envelope proteins, responses to two panels of gp120 antigens were examined two weeks after the fourth vaccination (Fig. S3). One panel included ten antigens from clade C viruses isolated in Southern Africa and India. Another panel included eight antigens from clades A, B, AE, and BC viruses (17). There were no significant differences in the comparison of the area under the curve (AUC) measures between the ID groups with and without pIL-12 or between the ID and IM groups; however, for both antigen panels, the AUC for the ID + IL-12 group (red curve) was generally greater than that for the other groups, especially at the higher magnitudes. Statistical comparisons performed for each antigen separately revealed three clade C antigens with significantly higher response rates for ID compared to IM (96ZM651.D11gp120.avi, CAP210_D11gp120.avi/293F, TV1c8_D11gp120.avi/293F) and two antigens with significantly higher magnitude for ID compared to IM (clade AE A244 D11gp120.avi, clade B TT31P.2792_D11gp120.avi/293F, $p \leq 0.04$, $q \leq 0.15$).

The RV144 HIV vaccine trial is the only study to show efficacy against HIV infection (18).

Binding IgG antibody levels to the variable regions 1 and 2 (V1V2) of HIV-1 Env were inversely correlated with risk of HIV infection (19). We therefore measured the levels of V1V2-specific
antibodies at two weeks and six months after the fourth vaccination (Fig. 4). IgG binding antibodies to V1V2 Env antigens were detected in up to 56% of participants two weeks after the fourth vaccination (clade AE shown in Fig. 4). Response rates were lower for the IM + IL-12 group compared to the ID + IL-12 group for all three antigens tested, but this difference was only significant for the clade AE antigen (14% vs. 56%, p=0.006, q=0.04). Response rates markedly decreased by six months after the fourth vaccination in all groups.

Antibodies with the same epitope specificity but of different subclasses can have different functional attributes. In particular there is precedence for the role of IgG3 antibodies in immune-mediated pathogen control, and in fact, we have shown that vaccine-induced Env V1V2 IgG3 correlates with lower HIV-1 infection risk in the RV144 vaccine efficacy trial (20). In our study, response rates were high for IgG3 sub-class antibodies binding to consensus gp140 and gp41 (Fig. 5), and low for consensus gp120 (Fig. S4). For both gp140 and gp41, response rates were significantly higher for the ID + IL-12 group compared to the IM + IL-12 group (p ≤ 0.01, q ≤ 0.05), although response magnitudes were not different. Response rates and magnitudes dropped significantly at six months after the fourth vaccination. IgG3 responses to gp120 and Gag p24 were only detected in a few individuals (Fig. S4). IgG3 responses to the V1V2 antigens were very low with the highest response rate (18%) for the ID + IL-12 group after the fourth vaccination, and nearly all responses not detectable by six months.

The functionality of the antibody responses was also determined by exploring the serum neutralization capacity, antibody dependent cellular phagocytosis (ADCP) and antibody dependent cellular cytotoxicity (ADCC). Neutralizing antibodies were mainly detected only to the tier 1A viral isolate, MW965.26, with response rates as high as 75% for the ID + IL-12 group two weeks after the fourth vaccination (Fig. 6A). Only one individual in the ID + IL-12 group had a response to a tier 1B virus (clade C DU156.12, titer 20.7) and to three tier 2 viruses (clade C 25710-2.43, titer 20.9, clade C Cell76_A3, titer 22.7, and clade G X1632-S2-B10, titer 12.4).
The ADCP response rate to the ConS gp140 antigen after the fourth vaccination was significantly higher in the ID + IL-12 group than in the IM + IL-12 group (82% vs. 50%, p=0.015) although the magnitude among responders did not differ between the groups (Fig. 6B). ADCC as measured using target cells infected with a clade C infectious molecular clone revealed few responses (Fig. 6C). In the ID + IL-12 group, responses were detected in 8% and 12% of participants after the third and fourth vaccinations, respectively. Two individuals had a response after the third vaccination, but no responses were detectable after the fourth vaccination. Results were similar for the assay using gp120-coated target cells with detectable responses in 16% of participants after both the third and fourth vaccinations for the ID + IL-12 group and for the IM + IL-12 group, one individual had a response, however this response was also present at baseline (Fig S5).

**Correlations between immunogenicity measures**

Correlation analyses were performed across the main immune measures for IgG binding antibodies, T-cell ICS, and ADCP. ADCC was not included since few responses were detected. Scatterplots are shown in Fig. S6 including Spearman correlation coefficients (r) displayed on a gradient color scale. The most significant correlations with the largest correlation coefficients are between different measures within the same class of response, such as different antibody measures or different CD4+ or CD8+ T-cell measures. There is relatively poor correlation between CD4+ and CD8+ T cells (highest r value is for CD4+ vs. CD8+ for any HIV protein, r=0.44). There is also not a strong correlation between the antibody measures and the T-cell measures (highest r value of 0.51). ADCP correlated well with IgG binding antibody responses, especially for Env gp120 (Con 6) and Env gp140 (Con S), with r values of 0.84 and 0.87, respectively. Thus, the antibody and T-cell measures appear to be relatively independent and provide unique information regarding immunogenicity, although the ADCP results may be predicted by the binding antibody data.
Discussion

The HVTN 098 study demonstrated excellent cellular and humoral immunogenicity for DNA vaccination delivered via CELLECTRA® EP. Although prior DNA vaccination studies have induced CD4+ T cells (4), the concomitant induction of HIV-specific CD8+ T cells in HVTN 098 in more than half of the vaccine recipients has been observed infrequently as in our prior study that administered DNA via IM and with pIL-12 and EP, HVTN 080 (6)). Furthermore, the generation of Env-specific antibodies in nearly all vaccine recipients has not been seen in previous HIV vaccine studies using DNA. The ID route of administration was equivalent or superior to administration by IM even at 1/5th the IM dose, when both were administered via EP and with pIL-12.

The effect of pIL-12 was evaluated only in the groups that received ID/EP (with and without pIL-12). Although the effect of pIL-12 was not as marked as the comparisons between route of administration, pIL-12 significantly improved the CD4+ T-cell response rate and accelerated the antibody response by achieving near maximal responses by the third vaccination, rather than the fourth vaccination. The increase in T-cell response is consistent with our prior study (HVTN 080) also showing increase in CD4+ T cell responses (although not statistically significant) when pIL-12 was included when the vaccine was administered EP (6).

However, another prior study (HVTN 087) that used DNA manufactured by a different company revealed the opposite effect, i.e., decreased CD4+ T cell responses when pIL-12 was included, perhaps suggesting that the specific design of the DNA plasmids is a critical factor for any beneficial effect of pIL-12 (16). Any beneficial effects on antibody response could not be evaluated due to the lack of induction of these responses in the prior studies. In light of the limited benefit observed in our current study and somewhat conflicting results in prior studies, further testing is needed to definitively document utility of pIL-12.

The remarkable binding antibody response to Env in a DNA-only vaccine regime is unique to our current study (HVTN 098) and was not observed in our prior study (HVTN 080).
that administered lower dose env DNA via IM/EP with pIL-12 (6). In that prior study, the CD4+ and CD8+ T-cell responses to Gag were similar to those in HVTN 098, but there was minimal cellular or antibody response to Env. For that reason, the novel env vaccine including two plasmids encoding clade A and C consensus immunogens, respectively, was designed and evaluated in this study. The env vaccine dose was also markedly increased from 1 mg to 6 mg in the IM group (3 mg for env A and 3 mg for env C). Since the IM + IL-12 group in HVTN 098 is otherwise comparable to the prior study (i.e., same EP device), the enhanced humoral immunogenicity observed must be due to either the env design or increased dose, or both. Because of the recognized importance of inducing antibody responses for any candidate HIV vaccine, whether neutralizing or non-neutralizing, our study demonstrates the potential of DNA to serve as a component of a prime-boost regimen or even as a single agent vaccine if functionality of induced antibodies can be further improved, such as for ADCC activity.

We explored the antibody profiles induced to evaluate responses that may be protective based on prior immune correlates studies. Similar to other HIV vaccine candidates tested to date, we only detected neutralizing antibodies to tier 1 viruses and little or no activity against the more difficult to neutralize tier 2 viruses. Antibodies to Env V1V2 and certain Fc-mediated antibody effector functions were shown to be a protective correlate against HIV acquisition in the RV144 vaccine trial (19-24). Though initially not a focus when the Env plasmids were designed, in HVTN 098, similar to RV144, V1V2-specific antibodies were induced in up to 56% of the ID + IL-12 vaccine recipients, but the durability of these responses by six months following the last vaccination was poor and requires improvement. Antibody-dependent cellular cytotoxicity (ADCC) was detected infrequently in vaccine recipients in this study, and the magnitudes were in a similar range of what we detected in other clinical trials. Therefore, future regimens could be modified to improve antibody functionality and binding specificity, perhaps by DNA design or by including a protein or viral vector boost to the DNA. Antibody-dependent cellular phagocytosis (ADCP) was detected in the majority of the ID + IL-12 and also in the IM +
IL-12 vaccine recipients. Although the potential clinical benefit of this function cannot be determined in this phase 1 study, ADCP was one of the antibody Fc effector functions associated with immune correlates in the RV144 trial and recently shown by our group to be a correlate of decreased HIV-1 risk in HVTN 505 (25). Further studies are needed with larger sample sizes to determine if ID delivery improved antibody Fc effector functions over IM delivery.

Unlike the antibody responses, both the CD4+ and CD8+ T-cell responses were maintained or slightly reduced six months after final vaccination. Although many of the antibody measurements were improved with ID delivery compared to IM, T-cell responses were similar between both routes. Comparing to other HIV vaccine regimens, the CD4+ T cell response rates in RV144 were under 50% with very few CD8+ responses detected (19), and for adenoviral (Ad)-based vaccines the CD8+ T cell responses were dominant such as for the Ad5 vaccine in the Step study (CD4+ T cells were induced in 41% and CD8+ T cells were induced in 73% of vaccine recipients) (26) and for the DNA/Ad5 prime boost used in HVTN 505 (62% and 64% CD4+ and CD8+ T cell responses, respectively) (27). Thus, CD4+ T cell response rates were the highest for the DNA-only vaccines we tested, but CD8+ T cell response rates were somewhat lower than the Ad5-containing regimens. Both the CD4+ and CD8+ T cells included high proportions of polyfunctional T cells, although cells expressing IL-4 were rarely detected. Since polyfunctional CD4+ T cells expressing IL-4 were a protective correlate in RV144, increased induction of these cells may be beneficial and may contribute to a more durable antibody response (28). The cellular immunogenicity differed markedly between Env, Gag and Pol, with the dominant CD4+ and CD8+ T-cell responses to Env. Gag-induced CD4+ T-cell responses in about half of vaccine recipients, but few, if any, CD8+ T-cell responses. Pol was poorly immunogenic in contrast to the high response in our prior HVTN 080 study (79% response rate for CD4+ T cells and 58% for CD8+ T cells) (6). The lower responses for Gag and Pol could simply be a dose effect since the combined dose for the env plasmids was six-
times the dose for the *gag* and *pol* plasmids, or due to the design of the *pol* plasmid.

Specifically, the removal of integrase from the *pol* vaccine in HVTN 098 for potential safety concern may result in some epitope loss, thus leading to lower Pol-specific cellular responses. Antigenic competition is also a possibility considering the dominant Env response (29). Since the ID route is dose sparing, it would be feasible to increase the *gag* and *pol* dose in future studies. Further optimization of the design of the *gag* and *pol* plasmids should also be considered.

Overall, HIV DNA vaccines have previously demonstrated limited immunogenicity lacking CD8+ T-cell and antibody induction and were initially considered only as one component of a combination vaccine regimen. In contrast, our study demonstrates that DNA alone (with adaptive EP and pIL-12) can induce broad immunity across both CD4+ and CD8+ T cells as well as antibody production. The ID/EP route is equivalent or superior to the IM/EP route, and the ID/EP route was shown to be less painful and thus more tolerable in this study (Edupuganti, unpublished observations). Given that the ID route is dose sparing, this increases the feasibility of using higher doses (although relatively low compared to what would be used in IM). Higher antigen doses may be necessary to improve durability of antibody responses and increase ADCC function in order to enhance potential efficacy, and further optimization of the plasmids and/or combination vaccination, including a boost using protein and/or viral vectors, could possibly achieve this goal. DNA has a number of logistical advantages in terms of design, manufacture and production as compared to other vaccine approaches for genomic delivery such as viral vectors and also as compared to protein/adjuvant approaches. Given the broad cellular and humoral immunogenicity now demonstrated, DNA has potential for use as a single agent vaccine modality in HIV as well as for a rapid response to emerging infections such as coronavirus, Zika, Ebola and new strains of influenza.
Methods

Study Design

Details for the study cohort, study agents, and study design are described elsewhere (Edupuganti et al., submitted) and are summarized here. The study schema is included as Table S1. Participants were healthy, HIV-uninfected adults, age 18-55 years. The study schedule consisted of four groups each receiving four vaccinations at months 0, 1, 3, and 6 administered via EP. Group 1 was a pilot safety group testing low-dose PENNVAX®-GP with plasmid IL-12 (pIL-12) DNA (n=5) or placebo (n=1) delivered via ID/EP. Due to the small size of this group, immunogenicity data for this group are not included in the analyses presented here.

Groups 2 and 3 received vaccinations via ID/EP: Group 2 without pIL-12 (referred to as “ID no IL-12”, n=20) or placebo (n=2), and Group 3 with pIL-12 (referred to as “ID + IL-12”, n=30) or placebo (n=3). Group 4 received vaccinations via IM/EP, with pIL-12 (referred to as “IM + IL-12”, n=30) or placebo (n=3). For this IM group, the doses of pIL-12 and the gag and pol plasmids were 1 mg each, and the dose of the clade A and C env plasmids were 3 mg each. For the ID administration in groups 2 and 3, 1/5th the IM dose of all HIV plasmids was used (0.2 mg each for gag and pol, and 0.6 mg for each env A and env C), and half the vaccine dose was administered at each of two injection sites (opposite deltoids). In addition, group 3 received 0.4 mg of pIL-12, split and administered across two injection sites.

The PENNVAX®-GP env consensus clade A and clade C plasmids included an optimized IgE leader sequence, designed to express and traffic protein to the surface of transfected cells. The gag and pol plasmids were consensus multiclade (A, B, C and D). IL-12 DNA consisted of a single plasmid containing a dual promoter system for expression of both the IL-12 p35 and p40 genes necessary for the production of the active heterodimeric IL-12 protein. The placebo consisted of sterile water. Two electroporation devices were used; the
CELLECTRA® 3P EP system was used for ID delivery and the CELLECTRA® 5P EP system was used for IM delivery (30, 31).

Sample Processing

Serum for humoral assays was obtained from serum-separating tubes (SSTs) and frozen at –80°C. Peripheral blood mononuclear cells (PBMCs) for cellular assays were isolated and cryopreserved from heparin-anticoagulated whole blood within six hours of venipuncture, as described previously (32). Samples were collected at two weeks after the third vaccination, as well as two weeks and six months after the fourth vaccination for the immunogenicity assessments described below.

Intracellular Cytokine Staining (ICS) Assay

Flow cytometry was used to examine HIV-1-specific CD4+ and CD8+ T-cell responses using a validated ICS assay. The assay was similar to a published report and the details of the staining panel are included in Table S2 (33). The peptide pools evaluated were global potential T-cell epitopes pooled by frequency with three pools for Env, one pool for Gag and two pools for Pol (34). Previously cryopreserved PBMC were stimulated for six hours with the six synthetic peptide pools. As a negative control, cells were not stimulated. As a positive control, cells were stimulated with a polyclonal stimulant, staphylococcal enterotoxin B (SEB). Two replicates were tested for the negative control. CD4+ and CD8+ T cells expressing IFN-γ and/or IL-2 were the primary immunogenicity endpoints. Since the multiple pools for each HIV protein can include variant peptides covering the same protein region, the overall response for Env and Pol (referred to as Any Env and Any Pol) was represented as the maximum of each of the pools for each protein (see Table S3). The overall response to “Any HIV protein” was defined as the sum of the response to Any Env, Any Pol and the response to Gag.

Several criteria were used to determine if assay data were acceptable and could be statistically analyzed. The blood draw date must have been within the allowable visit window as
determined by the protocol. On the second day after sample thawing, the viability must have been 66% or greater. If not, the sample for that specimen at that timepoint was retested. If upon retesting the viability remained below this threshold, the ICS assay was not performed and no data were reported to the statistical center for the timepoint. For the negative control acceptance criteria, if the average cytokine response for the negative control wells was above 0.1% for either the CD4+ or CD8+ T cells, then the sample was retested and the response for the retested sample was analyzed. Furthermore, the total number of CD4+ and CD8+ T cells must have exceeded certain thresholds. If the number of CD4+ or CD8+ T cells was less than 5,000 for any of the HIV-1 peptide pools or one of the negative control replicates for a particular sample, data for that stimulation were filtered. If both negative control replicates were <5,000 cells, the sample was retested. If upon retesting, one negative control replicate was <5,000, the negative control replicate with >5,000 cells was used. If both negative control replicates from the retest for a T-cell subset were <5,000, then data for the T-cell subset were not included in the analysis.

Positivity was determined using a one-sided Fisher's exact test (35). A multiplicity adjustment was made across all tested HIV antigens using the discrete Bonferroni adjustment method. If the adjusted p-value for an antigen was ≤10e-5, the response to the antigen for the T-cell subset was considered positive. If any peptide pool was positive for a T-cell subset, then the overall response for that T-cell subset was considered positive.

T-cell Polyfunctionality Analyses

COMPASS (Combinatorial Polyfunctionality Analysis of Single Cells) is a computational framework for unbiased polyfunctionality analysis of antigen-specific T-cell subsets (28). Participant-level responses were quantified by two summary statistics: 1) the functionality score (FS) is defined as the estimated proportion of Ag-specific subsets detected among all possible T-cell subsets; 2) the polyfunctionality score (PFS) is similar, but weighs the different subsets by
their degree of functionality. For this analysis, expression of IFN-γ, IL-2, IL-4, IL-17a, TNF-α, CD40L, and granzyme B were included for the CD4+ T cells and the same markers, except for CD40L, were included for CD8+ T cells. Scores were compared between treatment groups using the Wilcoxon rank sum test. For Env-specific scores, overall responses across pools were computed by summing the cell counts, both for the number of expressing cells and the total cell count (numerator and denominator), in fitting a COMPASS model. A heatmap for each stimulation and T-cell subset shows the mean posterior probabilities of antigen-specific responses from COMPASS.

**Binding Antibody Multiplex Assays (BAMA)**

Serum HIV-1 specific IgG and IgG3 responses were measured on a Bio-Plex instrument (Bio-Rad) using a standardized custom HIV-1 Luminex assay (20, 36) that uses gp120 and gp140 proteins and V1V2 antigens described previously (17, 37). Details of the antigens included are listed in Table S3. The positive control was purified polyclonal IgG from HIV subjects (HIVIG) using a 10-point standard curve (4PL fit). The negative controls were HIV-1 seronegative human sera and blank beads.

Several criteria were used to determine if data from an assay were acceptable and could be statistically analyzed. First, the blood draw date must have been within the allowable visit window as determined by the protocol. Second, if the blank bead negative control exceeded 5,000 MFI, the sample was repeated. If the repeat value exceeded 5,000 MFI, the sample was excluded from analysis due to high background. Samples from post-enrollment visits were declared to have positive responses if they met three conditions: (1) the MFI minus blank values were ≥ antigen-specific cutoff at the 1:50 dilution level (based on the 95th percentile of the baseline visit serum samples and at least 100 MFI), (2) the MFI minus blank values were greater than 3 times the baseline (day 0) MFI minus blank values, and (3) the MFI values were greater than 3 times the baseline MFI values. The MFI minus blank responses (MFI*) at the
1:50 dilution level was used to summarize the magnitude at a given timepoint. For the expanded gp120 panels shown in Fig. S3, simultaneous evaluation of magnitude and breadth was used as previously described(38).

HIV-1-Specific Neutralizing Antibody Assays

Neutralizing antibodies against HIV-1 were measured as a function of reductions in Tat-regulated luciferase (Luc) reporter gene expression in TZM-bl cells (39). The assay measured neutralization titers against Env-pseudotyped viruses that exhibit a tier 1A, tier 1B or tier 2 neutralization phenotypes (see Table S3 for additional details on the antigens used). Response to a virus/isolate in the TZM-bl assay was considered positive if the neutralization titer was above a pre-specified cutoff (one-half the lowest dilution tested). A titer was defined as the serum dilution that reduces relative luminescence units (RLUs) by 50% compared to the RLUs in virus control wells (cells + virus only) after subtraction of background RLU (cells only). The pre-specified cutoff was 10 for TZM-bl cells.

Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC)

ADCC-mediated antibody responses were measured by two assays. The luciferase assay used clade A and clade C infectious molecular clone (IMC)-infected target cells (see Table S3 for details on the antigens used). Participant sera in addition to control sera were incubated with IMC-infected cells and tested in a 96-well plate. ADCC was detected through the use of Viviren luminescence. One positive control in duplicate and one standardized negative control in duplicate were used per plate. The readout is reduction in relative luminescence units (RLU) referred as percentage specific killing (40, 41). For each sample, percent specific killing was measured in two wells at six dilution levels: 1:50, 1:250, 1:1250, 1:6250, 1:31,250 and 1:156,250. For each subject at each timepoint, percent loss of luciferase activity was calculated relative to control wells for each experimental well and averaged over wells within subject, timepoint, and dilution. The analyses in the data focused on the following
readouts based on the baseline-subtracted percent loss of activity: (1) peak percent loss of luciferase activity defined as the maximum activity across the six dilution levels (“peak activity”), and (2) nonparametric area under the percent loss of activity vs. log10(dilution) curve (“AUC”), calculated using the trapezoidal rule, with activity less than 0% set to 0%. A positive response is defined as peak baseline-subtracted activity greater than or equal to 10% and baseline-subtracted activity greater than or equal to 10% for either the 1:50 or 1:250 dilution.

A second ADCC assay, the GranToxiLux (GTL) assay, tested against clade A, B and C Env using gp120-coated cells (percent granzyme B readout) (see S3 Table for details on the antigens used) (42). Participant sera were incubated with effector cells and gp120-coated target cells and ADCC was quantified as net percent granzyme B activity, which is the percent of target cells positive for GranToxiLux (GTL) detected by flow cytometry. For each participant at each timepoint, percent granzyme B activity was measured at six dilution levels: 1:50, 1:250, 1:1250, 1:6250, 1:31,250 and 1:156,250 for each antigen. The analyses in the data focused on the following readouts: (1) peak net percent granzyme B activity defined as the maximum activity across the six dilution levels (“peak activity”), and (2) nonparametric area under the net percent granzyme B activity vs. log10 (dilution) curve (“AUC”), calculated using the trapezoidal rule. Peak activity less than 0% was set to 0%. A positive response was defined as peak activity greater than or equal to 8%.

**Antibody-Dependent Cellular Phagocytosis (ADCP)**

To assess the ability of vaccine-induced antibodies to engage FcR, ADCP was measured in serum specimens obtained at baseline and two weeks after fourth vaccination in the ID + IL-12 and IM + IL-12 treatment groups, the two groups with the better immunogenicity in the other assays, using methods previously described (43). Briefly, neutravidin fluorescent beads were coated with the ConS gp140 biotinylated HIV-1 antigen, then incubated with monoclonal antibodies (positive control CH31 and negative control CH65) or diluted participant serum (1:50 dilution). For the ADCP assay, THP-1 cells (pre-treated with anti-human CD4 to
reduce CD4-Env mediated virus internalization) were incubated with the antibody/bead mixture then paraformaldehyde-fixed before analysis by flow cytometry. A phagocytic score was determined based on the ratio of experimental sample to the no antibody control. The mean phagocytosis score was defined as: (% bead positive for participant x MFI bead positive for participant) / (% bead positive for the no antibody control x MFI bead positive for the no antibody control). Samples were assayed in duplicate and the average scores of the replicates were reported. This ADCP assay has been standardized, but has not been qualified or validated.

Samples from post-baseline visits were declared to have positive responses if both of the following criteria were met: mean phagocytosis score ≥ 1.6, and mean phagocytosis score ≥ 3x the mean phagocytosis score at baseline. The positivity threshold, 1.6, is defined separately for each protocol and antigen as the 95th percentile of the mean phagocytosis score of 60 baseline samples.

**Statistics**

Positive response rates were compared using the Fisher’s exact test for unpaired data (between treatment groups) and the McNemar test for paired data (between visits). Response magnitudes among positive responders were compared using the Wilcoxon rank sum test for unpaired data and the Wilcoxon signed rank test for paired data. All p values are two-sided. False-discovery rate adjusted q values were calculated to account for multiple antigens, multiple timepoints, or treatment groups (when appropriate) (44). Q values ≤ 0.2 with p value < 0.05 were considered significant for the purpose of hypothesis generating. The q values (i.e., adjusted p values) are listed in the figures for all comparisons for which the unadjusted p value was < 0.05.

**Study Approval**

The study was reviewed and approved by the Institutional Review Boards at the Fred Hutchinson Cancer Research Center, University of Rochester, Vanderbilt University and Emory
University. Written informed consent was received from participants prior to inclusion in the study.

**Author Contributions**

SCD, SE, ME, YH, ES, LP, SAK, MP, AK, NYS, MLB, DBW, LC, and MJM contributed to the concept, design, and implementation of this clinical trial. SAK, MCK, JM, and MJM contributed to the clinical conduct of this trial. MCW, JY, MPM, ASK, JDB, LH, SW, NYS MLB, and DBW contributed to the design and preliminary evaluation of the study products. SCD, GF, GDT, DCM and MJM contributed to the immunogenicity testing. YH, XH, and YL contributed to the statistical analysis. All authors contributed to the writing and review of the manuscript.

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References


Fig. 1. HVTN 098 CONSORT diagram.
Fig. 2. T-cell responses measured by intracellular cytokine staining (ICS). (A) CD4+ and CD8+ T-cells expressing IFN-γ and/or IL-2 to any HIV peptide pool. Positive responses are shown in filled circles in color, negative responses are shown in open gray triangles. Box-plots represent the distribution for the positive responders only. Bar plots show response rates. Numbers below the bars indicate numbers of positive responders and total participants. Assays were performed two weeks after the third and fourth vaccinations and six months after the fourth vaccination. (B) COMPASS CD4+ and CD8+ T-cell polyfunctionality scores (PFS) to any HIV Env peptide pool. (C) Heatmaps for CD4+ and CD8+ T-cell responses to any Env peptide pool showing the mean posterior probabilities of antigen-specific responses from COMPASS. Columns correspond to the different subsets of cytokines being considered grouped from left to right by increasing number of functions in the key below the heatmap; a filled box indicates cells in that column are expressing that function with different colors indicating cells with 1, 2, 3, 4 or 5 functions. Rows correspond to mean across the individual participants in each treatment group at each timepoint. Each cell shows the probability that the corresponding antigen-specific subset (column) is being expressed in the corresponding treatment group in average (row), and is color-coded ranging from white (zero) to dark purple (one). Positive response rates were
compared using the Fisher’s exact test for unpaired data (between treatment groups) and the McNemar test for paired data (between visits). Response magnitudes among positive responders were compared using the Wilcoxon rank sum test for unpaired data and the Wilcoxon signed rank test for paired data. All p values are two-sided. False-discovery rate adjusted q values were calculated to account for multiple antigens, multiple timepoints, or treatment groups.
Assays were performed two weeks after the third and fourth vaccinations and six months after the fourth vaccination. Positive responses are shown in filled circles in color, negative responses are shown in open gray triangles. Box-plots represent the distribution for the positive responders only. Bar plots show response rates. Numbers below the bars indicate numbers of positive responders and total participants. Positive response rates were compared using the Fisher’s exact test for unpaired data (between treatment groups) and the McNemar test for paired data (between visits). Response magnitudes among positive responders were compared using the Wilcoxon rank sum test for unpaired data and the Wilcoxon signed rank test for paired data. All p values are two-sided. False-discovery rate adjusted q values were calculated to account for multiple antigens, multiple timepoints, or treatment groups.
Fig. 4. IgG binding antibody responses as measured by binding antibody multiplex assay (BAMA) against clade AE (A244) Env V1V2. Positive responses are shown in filled circles in color, negative responses are shown in open gray triangles. Box-plots represent the distribution for the positive responders only. Response rates are listed above each graph along with numbers of positive responders and total participants. Positive response rates were compared using the Fisher's exact test for unpaired data (between treatment groups) and the McNemar test for paired data (between visits). Response magnitudes among positive responders were compared using the Wilcoxon rank sum test for unpaired data and the Wilcoxon signed rank test for paired data. All p values are two-sided. False-discovery rate adjusted q values were calculated to account for multiple antigens, multiple timepoints, or treatment groups.
Fig. 5. IgG3 binding antibody responses as measured by binding antibody multiplex assay (BAMA) against consensus Env gp140 and Env gp41. Positive responses are shown in filled circles in color, negative responses are shown in open gray triangles. Box-plots represent the distribution for the positive responders only. Bar plots show response rates. Numbers below the bars indicate numbers of positive responders and total participants.

Positive response rates were compared using the Fisher’s exact test for unpaired data (between treatment groups) and the McNemar test for paired data (between visits). Response magnitudes among positive responders were compared using the Wilcoxon rank sum test for unpaired data and the Wilcoxon signed rank test for paired data. All p values are two-sided.

False-discovery rate adjusted q values were calculated to account for multiple antigens, multiple timepoints, or treatment groups.
Fig. 6. Antibody functional responses. (A) Neutralizing antibody responses to Tier 1A Env-pseudotyped virus MW965.26 as determined by the TZM-bl neutralization assay two weeks after the third and fourth vaccinations. (B) Antibody-dependent cell mediated phagocytosis (ADCP) two weeks after the fourth vaccination. (C) Antibody-dependent cell mediated cytotoxicity (ADCC) as determined by the infected cell target assay for a clade C viral isolate (TV1). For all plots, positive responses are shown in filled circles in color, negative responses are shown in open gray triangles. Box-plots represent the distribution for the positive responders only. Response rates are listed above each graph along with numbers of positive responders and total participants. Response magnitudes among positive responders were compared using the Wilcoxon signed rank test; p values is two-sided.
Table 1. Response rates\textsuperscript{A} for T cells expressing IFN-\( \gamma \) and/or IL-2 at two weeks after the fourth vaccination.

<table>
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<tr>
<th>T cell</th>
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<th>ID no IL-12</th>
<th>ID + IL-12</th>
<th>IM + IL-12</th>
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<td>96%</td>
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<td>9/16</td>
<td>27/28</td>
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<td></td>
<td></td>
<td></td>
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<tr>
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<td>64%</td>
<td>44%</td>
</tr>
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<td>9/16</td>
<td>18/28</td>
<td>12/27</td>
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<td></td>
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\textsuperscript{A}Percentages above 0 are bolded.