Enhanced Immunogenicity of an HIV-1 DNA Vaccine Delivered with Electroporation via Combined Intramuscular and Intradermal Routes

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ABSTRACT
It is accepted that an effectively prophylactic HIV-1 vaccine is likely to have the greatest impact on viral transmission rates. As previous reports have implicated DNA-priming, protein boost regimens to be efficient activators of humoral responses, we sought to optimize this regimen to further augment vaccine immunogenicity. Here we evaluated single versus concurrent intradermal (i.d.) and intramuscular (i.m.) vaccinations as a DNA-priming strategy for their abilities to elicit humoral and cellular responses against a model HIV-1 vaccine antigen, CN54-gp140. To further augment vaccine-elicted T and B cell responses, we enhanced cellular transfection with electroporation and then boosted the DNA-primed responses with homologous protein delivered subcutaneously (s.c.), intranasally (i.n.), i.m., or transcutaneously (t.c.). In mice, the concurrent priming regimen resulted in significantly elevated gamma interferon T cell responses and high-avidity antigen-specific IgG B cell responses, a hallmark of B cell maturation. Protein boosting of the concurrent DNA strategy further enhanced IgG concentrations but had little impact on T cell reactivity. Interestingly protein boosting by the subcutaneous route increased antibody avidity to a greater extent than protein boosting by either the i.m., i.n., or t.c. route, suggesting that this route may be preferential for driving B cell maturation. Using an alternative and larger animal model, the rabbit, we found the concurrent DNA-priming strategy followed by s.c. protein boosting to again be capable of eliciting high-avidity humoral responses and to also be able to neutralize HIV-1 pseudoviruses from diverse clades (clades A, B, and C). Taken together, we show that concurrent multiple-route DNA vaccinations induce strong cellular immunity, in addition to potent and high-avidity humoral immune responses.

IMPORTANCE
The route of vaccination has profound effects on prevailing immune responses. Due to the insufficient immunogenicity and protection of current DNA delivery strategies, we evaluated concurrent DNA delivery via simultaneous administration of plasmid DNA by the i.m. and i.d. routes. The rationale behind this study was to provide clear evidence of the utility of concurrent vaccinations for an upcoming human clinical trial. Furthermore, this work will guide future preclinical studies by evaluating the use of model antigens and plasmids for prime-boost strategies. This paper will be of interest not only to virologists and vaccinologists working in the HIV field but also to researchers working in other viral vaccine settings and, critically, to the wider field of vaccine delivery.

To date, most licensed vaccines are based on the generation of neutralizing antibodies which are effective against invariant antigen-bearing pathogens. However, as antigenic variability increases, the number of licensed vaccines that are effective dramatically decreases (1). As a consequence, HIV-1, a retrovirus with exceptionally high antigenic variability, may require a completely novel vaccination strategy. Hence, in an attempt to augment vaccine-induced anti-HIV-1 T helper and antibody responses, we utilized three distinct concepts to formulate a novel immune-priming paradigm to precede protein boost vaccination. Specifically, we utilized (i) a DNA plasmid vector called Auxo-GTU, previously described to induce strong and durable T cell responses, in combination with (ii) electroporation (EP) and (iii) concurrent intradermal (i.d.) and intramuscular (i.m.) vaccinations.

The Auxo-GTU technology is a nonreplicating plasmid vector which utilizes the bovine papilloma virus type 1 (BPV1) transcription activator, the segregation/partitioning factor E2 protein, and its multimeric binding sites (2, 3). This has been shown to result in the enhanced transcriptional activity of the transgenes along with the potential for increasing the number of cells expressing the transgene (3). Furthermore, it has previously been utilized in clinical and preclinical studies and has been shown to display a good safety profile (4). DNA-based vaccination is an attractive mode of vaccine delivery. DNA vaccines utilize the host for in vivo biosynthesis of transgene products (5), hence imitating infectious pathways, and through host cell posttranslational modifications, the transgene products more accurately represent the conformation of naturally expressed viral antigens (6). Despite the many advantages, most conventional DNA vaccination strategies appear to be
poorly immunogenic. Therefore, DNA vaccines have failed to
translate from earlier murine studies to humans, leading to poor
efficacy in human clinical trials (7, 8), and as a consequence, no
prophylactic DNA vaccine is clinically approved for use in hu-
mans (5). To enhance the immunogenicity of DNA vaccines,
strategies such as promoter selection, codon optimization, and
different routes of administration have been employed (5). How-
ever, the delivery of DNA in association with EP has been shown to
dramatically increase gene expression and vaccine-induced re-
sponses over and above those that have been obtained by the use of
most existing adjuvant technologies (9–12).

Other aspects may play a significant role in the as yet limited
immunogenicity of DNA vaccines. For instance, while most vac-
cinations, including DNA, are delivered via the i.m. route (13), the
lower number of antigen-presenting cells (APCs) within muscle
tissues has been suggested to be a factor contributing to reduced
efficacy (2, 5, 14). i.m. vaccination has been described to result in
poor antigen-dependent T cell activation owing to the lack of
APCs in muscle tissue (14). Certainly, previous studies have
shown that i.d. vaccination increases the magnitude of multifunc-
tional CD4+ T cell responses compared to that achieved with i.m.
vaccination (15). Therefore, despite myocytes being good trans-
fection candidates (5), the location may be suboptimal for DNA-
based immune activation. As opposed to previously published
findings from studies evaluating single routes of DNA administra-
tion (16), here we report on the use of concurrent multiple-route
vaccination and inoculation i.d., a route that allows the vaccine to
reach a site with a plentiful and diverse number of APCs (17),
combined with concurrent i.m. vaccination. We hypothesized
that this dual-route vaccination may result in enhanced immune
responses to the in vivo-expressed plasmid DNA vaccine trans-
genes. Here we report that combination of the concurrent DNA
vaccination strategy (i.d. [EP] and i.m. [EP]) with a homologous
protein boost vaccination regimen enhances the levels of produc-
tion of antibody with a higher antigen-specific binding avidity.
Furthermore, we show that concurrent DNA priming can aug-
ment the vaccine-elicited T cell response, a factor critical for pro-
viding the necessary T cell help to B cells. Finally, we demonstrate
that the optimized DNA-priming, protein boost regimen can
translate to a larger animal model and drive the production of
neutralizing antibodies against different HIV-1 strains. To our
knowledge, this is the first report of an optimized concurrent par-
terental approach utilizing DNA EP in a prime-boost regimen to
achieve enhanced immune responses against the HIV-1 envelope.

MATERIALS AND METHODS

Plasmids, protein, and peptides. The HIV-1 vaccine construct was devel-
oped and prepared using the Auxo-GTU vector technology. The HIV-1
CN54-gp140 clade C/B gene (codon optimized) was inserted into the
Auxo-GTU vector backbone and DNA vaccine produced by Fit Biotec,
Finland. The recombinant CN54-gp140 protein was purchased from
Polymun Scientific (Austria). The gp140-stimulatory peptides, 15-mers
overlapping by 11 amino acids, were devised, synthesized (Insight Bio-
technology, United Kingdom), and split into two pools.

Immunizations. Six- to 8-week old female BALB/c mice (n = 8; Har-
lan, United Kingdom) were handled and procedures were performed in
accordance with the terms of a project license granted under the United
Kingdom Home Office Animals (Scientific Procedures) Act 1986. Mice
received three or four plasmid DNA (GTU-CN54-gp140) immunizations
at 3-week intervals as either i.d. (10 μg DNA in the lower back), i.m. (50
μg in the quadriceps), or i.d. and i.m. combination vaccinations. Injection
sites then received EP using either 5-mm or tweezer electrodes using an
ECM 830 square-wave electroporation system (BTX). Pulses consisted of
100 V of positive and negative polarity at a rate of 1 pulse/s, with each
pulse lasting 50 ms. Protein boost studies involved either two subcutane-
ous (s.c.), intranasal (i.n.), transcutaneous (t.c.), or i.m. protein boosts
(20 μg) using the CN54-gp140 protein in the absence of adjuvant. New
Zealand White rabbits (n = 8; Harlan, United Kingdom) were handled
and procedures were performed in accordance with the terms of a project
license granted under the United Kingdom Home Office Animals (Sci-
cific Procedures) Act of 1986. Rabbits were placed into three groups.
Groups 1 and 2 received three i.d. (EP) and i.m. (EP) DNA-priming vac-
cinations, while groups 1 and 3 received two s.c. recombinant gp140 pro-
tein boost vaccinations. For i.d. and i.m. vaccinations, 20 and 100 μg of
DNA were used, while 40 μg protein was used for s.c. protein boosts.

Sampling and processing. Blood was collected and centrifuged at
1,000 × g for 20 min. The serum was harvested and stored at −20°C. To
assess gamma interferon (IFN-γ) T cell responses, cultures of lympho-
cytes from spleens were prepared as described previously (18, 19).

Bioluminescence imaging. Real-time bioimaging of i.d. and i.m.
DNA vaccinations was performed using a multispectral Carestream in-
vivo FX Pro system. Briefly, either 50 μg (i.m.), or 30 μg (i.d.) GTU-Luc-
GFP plasmid was administered with or without EP to anesthetized BALB/c mice. Luciferase activity was measured 15 min after i.p. adminis-
tration of r-luciferin (150 mg/kg of body weight; Promega, United King-
dom). Bioluminescence images were acquired and analyzed using Car-
estream software and 60-s exposures.

Immunoglobulin ELISA and avidity assay. A quantitative immuno-
globulin enzyme-linked immunosorbent assay (ELISA) protocol de-
scribed previously (18, 19) was followed. Briefly, 5 μg/ml gp140-coated
ELISA plates were blocked with 200 μl/well 1% bovine serum albumin
and 0.05% phosphate-buffered saline (PBS)–Tween 20 (PBST). After
washing, samples were diluted and added to the plates for 1 h before
washing and addition of a 1:4,000 dilution of either anti-mouse IgG–
horseradish peroxidase (HRP), IgGl-HRP, IgG2a–HRP, or IgA–HRP
(Southern Biotech). Samples were developed using tetramethylbenzidine
(TMB), and the reaction was stopped using Stop solution (Insight Bio-
technologies, United Kingdom). Standards consisted of coating with anti-
mouse kappa (1:3,200) and lambda (1:3,200) light chains (Serotec, United
Kingdom), blocking as described above, and then addition of purified IgG
(Southern Biotech, United Kingdom) or IgA (Southern Biotech, United
Kingdom) starting at 200 ng/ml. The absorbance was read on a spectro-
photometer (VersaMax; Molecular Devices) using SoftMax Pro GxP (v5)
software. For the rabbit antigen-specific IgG ELISA, a protocol similar to
that described above was followed. For standards, plates were initially
coated with a 1:125,000 dilution of 2 mg/ml goat anti-rabbit IgG (Millipore,
United Kingdom), followed by 1/5 dilutions of purified rabbit IgG (1,000
ng/ml; Serotec, United Kingdom), and rabbit IgG was detected using
monoclonal anti-rabbit gamma chain-specific IgG (clone RG-96; Sigma,
United Kingdom).

The avidity of serum immunoglobulin was determined by the anti-
body-antigen binding resistance to 8 M urea. Samples were prediluted
to give an optical density (OD) at 450 nm (OD450) readout of between 1.0
and 1.5 and were added to gp140-coated plates. The plates were then
washed three times with either PBST or 8 M urea in PBST, before incu-
bating with anti-mouse IgG–HRP. Samples were developed with TMB as
described above. The avidity index (in percent) was calculated as the
OD450 of urea-treated samples/OD450 of PBST-treated samples. Antisera
with index values exceeding 50% were ascribed a high avidity, those with
index values of 30 to 50% were ascribed intermediate avidity, and those
with index values of <30% were ascribed a low avidity.

IFN-γ antigen-specific ELISPOT assays. IFN-γ enzyme-linked immu-
nosorbent spot (ELISPOT) assays (Mabtech, United Kingdom) were car-
ried out on splenocytes per the manufacturer’s instructions and as
described previously (18, 19). Plates were blocked for 30 min using complete
RPMI before addition of 5 × 106 cells/ml. Next, 5 μg/ml of gp140 peptide

Downloaded from http://jvi.asm.org/ on April 7, 2016 by Imperial College London
pools 1 and 2 was used to stimulate cells for 16 h to assess the responses. Unstimulated and 5 μg/ml phytohemagglutinin (Sigma, United Kingdom)-stimulated cells served as controls. To detect spots, biotinylated anti-IFN-γ antibody was added at 1 μg/ml for 2 h before washing and incubating with streptavidin-HRP for 1 h. Plates were washed as described above, and 100 μl/well of TMB substrate was added.

**Virus neutralization assay.** HIV-1 serum neutralization assays were performed using a luciferase-based assay in TZM.bl cells as described previously (20). Briefly, 3-fold serial dilutions of serum samples in 10% Dulbecco modified Eagle growth medium (100 μl/well) were performed in duplicate in 96-well plates. A 50-μl volume of virus was added to each well, and the plates were incubated for 1 h at 37°C. TZM.bl cells in growth medium (1 × 10^6 cells/well per 100-μl volume) containing 11 μg/ml DEAE-dextran (Sigma) were then added to each well. The neutralization assay used replicate wells of TZM.bl cells alone (cell control) and TZM.bl cells with virus (virus control). Following a 48-h incubation at 37°C, a 150-μl volume of assay medium was removed from each well and 100 μl of Bright-Glo luciferase reagent (Promega) was added. The cells were lysed for 2 min, before a 150-μl volume of cell lysate was transferred to a 96-well black solid plate, and luminescence was measured using a Victor 3 luminometer (PerkinElmer). The 50% inhibitory dose (ID_{50}) titer was calculated as the serum dilution that caused a 50% reduction in relative luminescence units (RLUs) compared to that for virus control wells after subtraction of cell control RLUs.

**Statistical analysis.** Statistical analysis of the data was carried out using a Mann-Whitney nonparametric t test and GraphPad Prism software.

**RESULTS**

**Concurrent i.d. and i.m. vaccination with EP enhances cellular responses as well as alters antibody avidity.** i.m. vaccination is the most common route for DNA vaccination. However, even though myocytes are highly susceptible to transfection, the response obtained by the targeting of muscle tissue for vaccine gene expression may be limited because of the low number of local APCs (21). This is in stark contrast to the response obtained by the targeting of skin, which is notable for its abundance of APCs. We therefore hypothesized that combining i.m. vaccination with concurrent i.d. vaccination might enhance vaccine-mediated humoral and cellular immune responses over and above the response normally seen by either route alone. Initially, we compared single-site i.d. or i.m. vaccination with concurrent i.d. and i.m. vaccination without EP (see Table S1 in the supplemental material). i.m. vaccination resulted in significantly (P = 0.0009) elevated levels of antibody in serum compared to those achieved by i.d. vaccination (Fig. 1a). However, both i.d. and i.m. vaccinations individually induced nonstatistically distinct IFN-γ spot-forming unit (SFU) responses (Fig. 1b), suggesting that individual i.d. vaccinations were equivalent to i.d. vaccinations for eliciting cellular responses. When comparing concurrent i.d. and i.m. vaccination with individual i.d. and i.m. vaccinations, the concurrent i.d. and i.m. vaccination regimen generated specific IgG concentrations similar to those generated by i.m. vaccination, and the specific IgG concentrations were significantly elevated compared to those achieved with i.d. vaccination (P = 0.0019) (Fig. 1a); however, the concurrent regimen increased antibody avidity compared to that achieved by i.m. vaccination (see Fig. S1a in the supplemental material). Furthermore, concurrent i.d. and i.m. vaccination significantly enhanced the T cell responses against peptide pool 1 (P = 0.003 and P = 0.014 compared to the responses for the individual i.m. and i.d. vaccination groups, respectively) and peptide pool 2 (P = 0.0379 compared to the responses for both the individual i.m. and i.d. vaccination groups) (Fig. 1b) relative to the responses seen for the individual i.m. or i.d. vaccination group, indicating that concurrent i.d. and i.m. vaccination is superior to i.m. vaccination with respect to avidity and the magnitude of cellular responses.

Next, we evaluated the impact of EP on concurrent i.d. and i.m. vaccination and the relative contribution that EP made to the cellular and humoral responses when applied to each vaccination site. All concurrent vaccination combinations generated equivalent and significant concentrations of serum IgG compared to those for the controls (P ≥ 0.0005) (Fig. 1c). As all the tested concurrent vaccination regimens elicited similar antibody titers, we utilized an avidity assay as a means to differentiate and qualitatively evaluate the humoral responses. Here we show that the i.d. (EP) and i.m. group and the i.d. (EP) and i.m. (EP) group generated the highest proportions of medium- and high-avidity responses in serum (Fig. 1f). Strikingly, the i.d. (EP) and i.m. (EP) regimen generated 50% more mice that seroconverted with a high-avidity response than the i.d. (EP) and i.m. regimen. This clearly demonstrates that the different concurrent vaccination regimens had different impacts on B cell maturation.

Interestingly, significant differences in mucosal IgG responses were observed. The i.d. and i.m. and the i.d. and i.m. (EP) regimens failed to generate significant quantities of IgG (Fig. 1d). However, the i.d. (EP) and i.m. (146 ± 42 ng/ml, P = 0.0006) and the i.d. (EP) and i.m. (EP) (107 ± 35.4 ng/ml, P = 0.0006) regimens elicited highly significant IgG responses in the vagina compared to those in the control and the i.d. and i.m. groups (P = 0.0024 and P = 0.0165, respectively). No significant difference was observed between the i.d. (EP) and i.m. and the i.d. (EP) and i.m. (EP) groups (Fig. 1d), suggesting that administration of EP via the i.d. route facilitates antibody localization in mucosal secretions. Evaluation of cellular responses revealed that all concurrent regimens elicited significant T cell responses (P ≥ 0.0005) to peptide pools 1 and 2 compared to the response by the controls (Fig. 1e). Furthermore, the i.d. (EP) and i.m. and the i.d. (EP) and i.m. (EP) regimens elicited significantly elevated T cell responses compared to the i.d. and i.m. and the i.d. and i.m. (EP) regimens for both peptide pool 1 (P = 0.003 and 0.001, respectively) and peptide pool 2 (P = 0.002 and 0.003, respectively). No significant differences in T cell responses were recorded between the i.d. (EP) and i.m. and the i.d. (EP) and i.m. (EP) groups (Fig. 1e). Taken together, these results show the concurrent i.d. (EP) and i.m. (EP) treatment regimen to be superior for eliciting strong humoral and cellular responses.

In addition to increasing transgene expression levels, EP has been described to act as an adjuvant through as yet undefined mechanisms (22–24). Therefore, using the empty vector, we compared the ability of EP delivered via the i.m. route (see Fig. S2a in the supplemental material) and EP delivered via the i.d. route (see Fig. S2b in the supplemental material) to alter antigen-specific immune responses. Using the empty vector, electroporation via the i.m. route either augmented or significantly increased cellular immune responses to the vaccine transgene delivered via the i.d. route. In contrast, this adjuvant activity was not observed when EP and empty vector were delivered via the i.d. route. Thus, suggesting that the route of EP delivery can significantly affect the ensuing cellular immune response.

**Concurrent DNA-priming (EP) and protein boost vaccinations enhance humoral responses.** DNA priming and subsequent protein boosting have been shown to significantly amplify humoral responses. Therefore, we assessed the ability of protein boost vaccination to augment the antibody responses elicited. We
FIG 1 Concurrent priming vaccination with EP elicits stronger cellular and higher-avidity antibody responses. (a and b) Single i.d. (10 μg) or i.m. (50 μg) vaccination was compared to concurrent i.d. and i.m. (10 μg and 50 μg) vaccination (n = 8 mice per group), in the absence of EP, for the elicited antigen-specific serum IgG antibody (a) and cellular (b) responses. Humoral and cellular responses were evaluated 1 week after the fourth and final vaccination by antigen-specific IgG ELISA and IFN-γ ELISpot assay and are shown as group means (SEM). (c to e) Comparison of the ability of different concurrent vaccination regimens, in the presence or absence of EP, to elicit antigen-specific serum (μg/ml) (c) and mucosal (ng/ml) (d) IgG responses and to stimulate systemic cellular immunity (numbers of SFU per million antigen-stimulated cells [SEM]) (e). Mice (n = 8 per group) were vaccinated 4 times at 3-week intervals, with bleeds occurring 1 week after each vaccination. Mucosal sampling occurred during study week 10, 1 week after the final vaccination. Statistical significance was assessed using the Mann-Whitney U test. *, P ≤ 0.05; **, P ≤ 0.005; ***, P ≤ 0.0005; N.S., not significant. (f) To assess antibody avidity, concurrent vaccination serum samples were prediluted to give an optical density of between 1 and 1.5 using an in-house antigen-specific ELISA. Samples were then titrated in an endpoint ELISA in duplicate using nonreducing (PBS) and reducing (8 M urea) washes after sample addition. Results are shown as the percent change in binding ([reducing OD/nonreducing OD] × 100).
evaluated the concurrent i.d. (EP) and i.m. (EP) DNA-priming strategy (here referred to as simply DNA) in combination with two subsequent protein boost vaccinations via the s.c., i.n., t.c., and i.m. routes (see Table S2 in the supplemental material). All prime-boost regimens generated significantly elevated IgG levels in the systemic compartment (Fig. 2a). The DNA vaccinations efficiently primed the immune responses, resulting in elevated IgG levels in all groups by the end of the 3rd priming (week 6). These responses were significantly amplified by all routes of protein boosting. DNA priming boosted by the s.c. (507 ± 60.5 μg/ml, P = 0.0003) and i.n. (566 ± 78.6 μg/ml, P = 0.0003) routes generated the highest IgG responses, closely followed by i.m. boosting (442 ± 62 μg/ml, P = 0.0047). Although the response was highly significant (119 ± 30 μg/ml, P = 0.0003), the DNA and t.c. protein boost regimen resulted in the lowest IgG profile. Serum IgA responses for all boost regimens were also significantly elevated compared to those for the protein controls (Fig. 2b). The DNA and i.n. route of protein boost vaccination was clearly superior to the other boost regimens for the production of antigen-specific IgA, resulting in highly significant serum IgA concentrations being recorded during study week 13 (3,107 ± 1.1 ng/ml, P = 0.0002). The DNA and s.c. (213 ± 83 ng/ml, P = 0.0002) and DNA and i.m. (196 ± 56.7 ng/ml, P = 0.0002) routes elicited similar levels of serum IgA; however, the DNA and t.c. (P = 0.029) regimen resulted in the least antigen-specific IgA being generated (Fig. 2b). Interestingly, however, the DNA and t.c. route was the

FIG 2 The route of administration of protein boost impacts the ensuing serum humoral responses. (a and b) Antigen (Ag)-specific IgG (a) and IgA (b) from serum were assessed (n = 8 per group) 1 week after three concurrent DNA-priming vaccinations (i.d. [10 μg] and i.m. [50 μg]) and two protein boosts (20 μg/dose). Protein boosts were delivered by either the s.c., i.n., t.c., or i.m. route. Antibody results are expressed as group means (μg/ml or ng/ml). Statistical significance was assessed using the Mann-Whitney U test. *, P ≤ 0.05; **, P ≤ 0.005; ***, P ≤ 0.0005. (c) To assess antibody avidity, vaccine serum samples were prediluted to give an optical density between 1 and 1.5 using an in-house antigen-specific ELISA. Samples were then titrated in an endpoint ELISA in duplicate using nonreducing (PBS) and reducing (8 M urea) washes after sample addition. Results are shown as the percent change in binding [(reducing OD/nonreducing OD) × 100].
only one that elicited elevated antigen-specific IgA responses after the final DNA-priming vaccination. Assessment of the serum antigen-specific IgG/IgA ratios revealed that the i.m. boosting route could dramatically alter the ratio of IgG/IgA, while the t.c. boosting regimen promoted IgG production over IgA production (Fig. 2c). The i.m. and s.c. routes produced almost equivalent ratios of IgG/IgA (Fig. 2c).

The impact of different protein boost vaccination routes on IgG avidity was assessed in order to ascertain the optimal prime-boost regimen. Interestingly, the DNA and s.c. route of boost vaccination outperformed all other boost regimens, generating the highest-avidity antibody responses (54.4%) (Fig. 2d). Furthermore, 62.5% of vaccinated animals in the DNA and s.c. group displayed high-avidity antibody binding, while the remaining animals displayed medium-avidity antibody binding. Interestingly, while the DNA and s.c. and the DNA and i.m. regimens generated similar IgG levels (Fig. 2a), the avidities of the elicited antibodies were markedly different (Fig. 2d). A noticeable reduction (15.7%) in avidity for the DNA and i.m. regimen compared to that for the DNA and s.c. regimen was recorded, clearly suggesting that the route of protein boost greatly impacts the quality of the antibody elicited. Furthermore, it is interesting to note that the DNA and i.m. (46%) regimen elicited antibody that had an avidity similar to that of antibody from the non-DNA-primed (46.2%) vaccinated controls, suggesting that although it is capable of immunologically priming for large quantities of antibody, DNA priming via the i.m. route had a minimal impact on B cell maturation (Fig. 2d).

Collectively, these results show that the s.c. route of protein boosting is preferential for the generation of high levels of serum antibodies with high avidity. The presence of protective antibody in external mucosal secretions is reasoned to add a significant layer of protection against HIV-1 infections occurring through susceptible mucosal surfaces. Therefore, we assessed the impact of the different protein boost vaccination regimens on vaginal antibody responses. All groups receiving DNA priming and protein boosting generated significant quantities of IgG in the vaginal vault compared to the quantities for the controls receiving protein only (see Fig. S3a in the supplemental material). In terms of the elicited vaginal IgA responses, only the DNA and i.m. protein boost vaccination group generated statistically significant responses (60 ± 28 ng/ml, P = 0.0025) (see Fig. S3b in the supplemental material). Hence, taken together, these data show that s.c., i.m., t.c., and i.d. protein boosting significantly augments mucosal IgG antibody responses, while only the i.m. route significantly increases the presence of mucosal IgA within external secretions.

The route of protein boost vaccination does not significantly alter the mean quantity of the vaccine-elicited cellular response. Next, we evaluated the impact of various routes of protein boost vaccination on the cellular response of DNA-primed mice. Here we show that all vaccine regimens which involved prior DNA immunizations, DNA and s.c. (840 ± 184 ng/ml, P = 0.0002), DNA and i.m. (1,113 ± 188 ng/ml, P = 0.0002), DNA and s.c. (1,018 ± 133 ng/ml, P = 0.0002), and DNA and i.m. (692 ± 137 ng/ml, P = 0.0002), generated highly significant cellular responses against peptide pool 1 compared to the responses for their respective controls (Fig. 3a). This was also seen for Env peptide pool 2, when the results obtained by the DNA and s.c. (595 ± 154 ng/ml, P = 0.0009), DNA and s.c. (702 ± 102 ng/ml, P = 0.0002), DNA and i.m. (1,106 ± 271 ng/ml, P = 0.0002), and DNA and i.m. (403 ± 88 ng/ml, P = 0.0002) regimens were compared to those for the controls (Fig. 3b). Comparisons between the regimens revealed no statistically significant difference between groups and, therefore, no advantage of one protein boost regimen over any other for augmenting total cellular immune responses.

Concurrent DNA (EP) and s.c. protein boost vaccinations elicit strong humoral responses and increase antibody avidity and HIV-1 neutralization in rabbits. The murine model is suboptimal for the assessment of neutralizing antibody responses due to high background levels of nonspecific inhibitor activity. Therefore, we extended our murine experiments into the larger rabbit model to test the antibody responses elicited in HIV-1 neutralization assays. New Zealand White rabbits were vaccinated using a triple DNA-priming (i.d. [EP] and i.m. [EP]) regimen followed by a double s.c. protein boost regimen (see Table S3 in the supplemental material). This was chosen on the basis of the observation that it induced the highest-avidity antibodies in the murine model. DNA priming utilized concurrent i.d. (20 μg) and i.m. (100 μg) vaccination and EP followed by homologous s.c. protein boosting (40 μg). The DNA and s.c. regimen (6,697.6 ± 2,547 μg/ml) elicited higher IgG responses than DNA (99.2 μg/ml ± 47.6) or protein alone (100.4 ± 44.1 μg/ml) (Fig. 4a), although all three vaccine regimens, DNA and s.c. (P = 0.0002), DNA and i.m. (P = 0.0167), and s.c. (P = 0.0167), elicited significant IgG responses compared to that in baseline control serum. Interestingly, triple i.d. (EP) and i.m. (EP) DNA vaccination induced antigen-specific antibody levels similar to those induced by the two s.c. protein vaccinations.

To better understand the elicited humoral response, we evaluated the avidity of serum from vaccinated rabbits. Here we found that after two protein boost vaccinations, the avidity index increased for all rabbits that had received prior DNA immunizations (Fig. 4b), suggesting that B cell maturation had occurred in rabbits and that after each protein boost vaccination, the avidity index increased in rabbits that had received prior DNA immunizations (Fig. 4b), suggesting that B cell maturation had occurred in rabbits and that after each protein boost vaccination, the avidity index increased in rabbits that had received prior DNA immunizations (Fig. 4b), suggesting that B cell maturation had occurred in rabbits and that after each protein boost vaccination, the avidity index increased in rabbits that had received prior DNA immunizations (Fig. 4b), suggesting that B cell maturation had occurred in rabbits and that after each protein boost vaccination, the avidity index increased in rabbits that had received prior DNA immunizations (Fig. 4b), suggesting that B cell maturation had occurred in rabbits 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**DISCUSSION**

Despite the overwhelming success of vaccines, there is a clear requirement for the immediate development of prophylactic interventions against a number of disease-causing organisms. This is particularly true for HIV-1, where vaccines are not available and current vaccine concepts have failed. Hence, novel vaccine technologies or paradigms will undoubtedly play an important role in protecting against HIV-1 acquisition. In this study, we used a DNA plasmid vector expressing gp140 as part of a preclinical study to deliver HIV-1 Env as a priming vaccine immunogen to
two different sites (by the i.d. and i.m. routes) concurrently. The aim was to elicit through rational vaccine design high-titer antibody and cellular responses over and above what is seen through conventional systemic single-site vaccination. While humoral antibody responses were the principal focus of this study, we also evaluated gp140-specific cellular responses, as CD4 T cell help is critical for not only the development of cytotoxic CD8 responses but also the development of Env-specific high-affinity neutralizing antibody responses. With that said, the results presented here clearly demonstrate that concurrent vaccinations are highly effective activators of both cellular and humoral responses. Of particular note is the i.d. (EP) and i.m. (EP) combination, which generated strong T and B cell responses, with the antibody elicited by this regimen demonstrating a higher avidity than antibody elicited by all other combinations tested. To our knowledge, this is the first time that a preclinical vaccination study has evaluated concurrent plasmid DNA vaccination as part of a prime-boost strategy, and this study suggests that concurrent DNA vaccine delivery may offer a new approach in HIV-1 prime-boost vaccine delivery research.

During each stage of evaluation, we selected the best vaccine combination on the basis of the elicited humoral and cellular responses. The optimized DNA regimen was then tested in a DNA-priming, protein boost protocol for any elicited virus-neutralizing activity. Prime-boost vaccination has previously been used to synergistically amplify memory T cell responses to vaccinating antigens (25) by invoking CD4+ T cells and corresponding T cell-dependent antibody responses (26). Furthermore, it is believed that DNA priming can facilitate memory B cell development, which can be selectively amplified upon recombinant protein boost vaccination and enhance antibody avidity (26). In this study, we detected a robust expansion in elicited antibody responses upon protein boosting in DNA-primed animals, with maximal affinity maturation being associated with the s.c. route. Hence, i.d. (EP) and i.m. (EP) vaccination with s.c. protein boosting was found to be the optimal vaccination regimen with respect to the magnitude and avidity of the systemic IgG responses. Curiously, while the s.c., i.n., and i.m. routes of protein boost vaccination elicited similar antigen-specific IgG concentrations in serum, the i.n. route elicited IgG responses which were the lowest in avidity (Fig. 2a and c), suggesting a lack of affinity maturation. This may be due to suboptimal stimulation of the ensuing immune responses through a combination of low antigen availability and a lack of Toll-like receptor activation. It is feasible to assume that i.n. protein boosting could result in the stimulation of germinal centers in an alternative repertoire of draining lymph nodes not normally associated with s.c., i.m., and t.c. vaccination. This could result in the de novo generation of antibody responses which have had less time for somatic hypermutation and affinity maturation. Hence, future studies evaluating the elicited T and B cell responses for affinity maturation and subsequent epitope breadth, as well as the impact of various coadministered adjuvants, could prove cru-
cial. Using the rabbit as a second animal model, we demonstrate increased antibody affinity with each subsequent s.c. protein boost following optimized DNA priming. This confirmed our earlier murine studies demonstrating that protein boosting augmented antibody avidity following prior DNA priming. Interestingly, previous studies in HIV infection models have correlated increased antibody avidity with lower viral titers at peak viremia (27), hence suggesting that B cell antibody maturation can play a protective role during HIV-1 infection. However, it is important to note that in previous studies, avidity maturation was ascribed to the use of adjuvants, such as granulocyte-macrophage colony-stimulating factor (27). Therefore, as we did not use any immunomodulatory adjuvant, we associate our increased avidity maturation to the optimized vaccination protocol.

The production of a neutralizing antibody response has been the goal for most prophylactic HIV-1 vaccines. This is especially true since a number of neutralizing antibody-passive infusion studies provided a proof of concept regarding protection against infection in the macaque model (28). Furthermore, the appearance of increased polyclonal serum avidity has been shown to coincide with the appearance of neutralizing antibody responses (29). However, the broadly cross-neutralizing antibody response thought to be required for protection against diverse strains of HIV-1 can take years to develop, if it develops at all (30). Thus, any vaccination strategy that preferentially drives the development of affinity maturation or at least speeds up the process may induce the production of protective neutralizing antibodies. By employing a sequential experimental process based on avidity, the humoral response, and cellular concentrations, we sought to harness the elusive vaccine-mediated neutralizing antibody response. Consequently, critical to our data was the ability of our vaccination regimen to elicit neutralizing antibody responses. The serum from the DNA-priming, protein boost group was able to neutralize viruses in both an intracranial and an interclade fashion, indicating that the elicited neutralizing antibody response had a degree of breadth. Currently, there is only one published phase 1 human clinical trial employing DNA-priming, protein boost vaccination (31). Within this particular study, the elicited antibody was capable of neutralizing tier 1 autologous pseudoviruses (31), hence providing compelling evidence that this type of vaccination regimen can translate effectively to humans. This is further supported by DNA-priming, protein boost vaccine regimens that elicited antibody responses capable of neutralizing homologous and heterologous viral strains in rhesus macaques (32). More recently, a coimmunization study was evaluated in mice and macaques (33). Here, the authors used a DNA and protein covaccination procedure and plasmids containing a mixture of Env and Gag DNA delivered by the i.m. (EP) route. The authors also administered gp120 protein formulated in EM-005 (glucopyranosyl lipid adjuvant-stable emulsion) to the same site. Interestingly, the authors reported that the concurrent regimen enhanced antibody responses and T cell polyfunctionality and had a broader cross-clade neutralizing potential (33).

Our current study did not use molecular adjuvants in order to clearly differentiate the impacts of the different components of the concurrent regimens. However, it would be interesting to evaluate the impact of adjuvants on the DNA-priming process as well as the protein boost to measure their impact on avidity and neutralizing potential. This may provide further enhancement, as adjuvants have been described to drive affinity maturation and promote antibody avidity against viral antigens (27, 34). As elicitation of HIV-1 broadly neutralizing antibody responses has been shown to be coincident with antibody affinity maturation as well as to possibly be driven by the presence of diverse simultaneously circulating forms of Env (35), it would be interesting to test the concurrent DNA-priming (i.d. [EP] and i.m. [EP]) and s.c. protein boost regimen with a multiclade vaccine formulation. Therefore, as two previous human clinical trials (VAX003 and VAX004) involving bivalent recombinant gp120s adjuvanted with alum have been shown to elicit tier 1 neutralizing antibody (36, 37), the adjuvan-

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REFERENCES


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