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Long-Term Maintenance of gp120-Specific Immune Responses by Genetic Vaccination with the HIV-1 Envelope Genes Linked to the Gene Encoding Flt-3 Ligand[1]

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DNA vaccines target dendritic cells (DC) to induce Ag-specific immune responses in animals. Potent HIV-specific immunity could be achieved by efficient priming of the immune system by DNA vaccines. We investigated a novel DNA vaccine approach based on the role of growth factors in DC expansion and differentiation. To this end, we constructed chimeric genes encoding the HIV envelope glycoproteins physically linked to the extracellular domain of Fms-like tyrosine kinase receptor-3 ligand (FLex; a DC growth factor; both mouse (m)FLex and human (h)FLex). These chimeric gene constructs synthesized biologically active, oligomeric FLex:gp120 fusion proteins and induced DC expansion ($CD11c^+CD11b^+$) when injected i.v. into mice. This DC expansion is comparable to that achieved by FLex DNA encoding native FLex protein. When delivered intramuscularly as DNA vaccines, hFLex:gp120 induced high frequencies of gp120-specific $CD8^+$ T cells in the presence or absence of FLex DNA-induced DC expansion, but gp120 and mFLex:gp120 elicited only low to moderate levels of Ag-specific $CD8^+$ T cells. In contrast, mFLex:gp120 induced high levels of anti-gp120 Abs under identical conditions of DNA vaccination. However, the Ab levels in mice immunized with DNA vaccines encoding hFLex:gp120 and gp120 proteins were low without DC expansion, but reached high levels comparable to that elicited by mFLex:gp120 only after the second boost in the presence of DC expansion. Importantly, the gp120-specific $CD8^+$ T cells persisted at high frequency for 114 days (16 wk) after a booster injection. These experiments provide insight into the importance of modulating DC function in vivo for effective genetic vaccination in animals. ***The Journal of Immunology,*** **2003, 170: 2496–2507.**

Deoxyribonucleic acid vaccines elicit both cellular and humoral immune responses and appear to predominantly target dendritic cells (DC)[4] for transgene expression in peripheral tissue sites for induction of T cell activation in secondary lymphoid organs (1–6). Despite limited expression of Ag in situ, impressive results have recently been obtained for DNA vaccines that are capable of priming the immune system more efficiently, resulting in highly boostable HIV-specific $CD8^+$ T cell responses in mice and rhesus macaques (7–13). As sentinels of the immune system, DC are at the crossroads of innate and adaptive immunity and therefore perform crucial roles in linking them functionally for immune augmentation. DC possess unique abilities to capture, process, and present Ags to T cells for inducing Ag-specific $CD8^+$ and $CD4^+$ T cell responses (14–16). This is effectively achieved by a complex series of events having specific requirements for optimal operation of DC in a cross-talk between components of innate and adaptive immunity (16).

The in vitro manipulation of DC has been shown to achieve a level of immunity in a number of tumor and infectious disease models, reinforcing the power of DC as the most efficient activators of immunity (17–20). However, it is most desirable to devise ways in which DC function could be modulated in vivo for the purposes of vaccination against infectious agents. Although DNA vaccines have the ability to induce both T cell and Ab responses, the magnitude, quality, and duration of these responses depend on a number of factors: Ags, injection sites, and delivery vehicles (3). In recent years, great strides have been made to optimize DNA vaccination protocols that take into account the functional properties of DC in immunity (3). For example, incorporation of CpG motifs (as activators of innate immunity) in DNA vaccines or as immunomodulatory nucleotides (oligodeoxynucleotides) have been shown to improve vaccine efficacy (3). Also, costimulatory molecules as vaccine adjuvants have been used to regulate DNA vaccine-induced immune responses in animals (3).

Because DNA vaccines target predominantly DC in vivo for induction of T cell immunity through direct or indirect mechanisms (5, 6), it is reasonable to hypothesize that increasing DC numbers would potentially translate into a more vigorous Ag-specific response. Many growth factors and cytokines play critical roles as soluble immune mediators in the orchestration of DC function in vivo (17–21). Of these many cellular factors, Fms-like tyrosine kinase receptor-3 ligand (Flt-3L) has the unique ability to expand the numbers of DC and NK cells in mice and humans (22–28). In an insightful study to our understanding of DC expansion in immunity, Pulendran et al. (29) have converted a tolerogenic peptide to one capable of inducing immune responses under conditions of increased DC numbers. Such Flt-3L-induced DC expansion has also been shown to mobilize distinct populations of

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[4] Abbreviations used in this paper: DC, dendritic cell; Flt-3L, Fms-like tyrosine kinase receptor-3 ligand; FLex, extracellular domain of Flt-3L; m, mouse; h, human; i.m., intramuscular(ly); ICC, intracellular cytokine staining; OPD, *o*-phenylenediamine; ER, endoplasmic reticulum; HSC, hemopoietic stem cell.

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DC in vivo, which might participate in eliciting T cell responses of different magnitude or quality. In the majority of the studies, recombinant Flt-3L protein (the extracellular domain of Flt-3L) was used to induce DC expansion (23, 24, 30, 31), and this DC expansion has been shown to enhance or modulate vaccine-induced immune responses against cancer and infectious agents in mice and humans (32–41).

In this study, we have conducted experiments to assess the efficacy of gp120 DNA vaccines having the extracellular domain of Flt-3L (FLex; mouse (m)FLex or human (h)FLex) covalently attached to them. We demonstrate that these chimeric genes direct the synthesis of hFLex:gp120 and mFlex:gp120 in vitro, which assemble as oligomers, and are also capable of inducing DC expansion in vivo when injected i.v. into mice. We further provide evidence that physical linkage of gp120 to FLex (at the N terminus) has modulating or enhancing effects on the generation of both Ag-specific $CD8^+$ T cell and Ab responses in mice.

Materials and Methods

DNA vaccine constructs

The expression vectors, pNGVL3-mFLex and pNGVL3-hFLex, coding for the extracellular domains of mouse or human Flt-3L were obtained from the National Gene Vector Laboratory (University of Michigan, Ann Arbor, MI). The *mFLex* and *hFLex* genes were fused to that coding for HIV-1 $gp120_{89.6P}$ or $gp140_{89.6P}$ (42) using the following primers in PCR (Fig. 1): 1) 5′-GGA ATT CAT GAC AGT GCT GGC GCC AGC CTG GAG C-3′; 2) 5′- CCC CAT AAT AGA CTG TGA CCC ACA AGC TAG CCC TGG GCC GAG GCT CTG GG-3′; 3) 5′-CCC AGA GCC TCG GCC CAG GGC TAG CTT GTG GGT CAC AGT CTA TTA TGG GG-3′; 4) 5′-ATG GAT CCT ACC AAT TCC ACA AAC TTG CCC ATT TA-3′; 5) 5′-ATG GAT CCT ATG CCC TGG TGG GTG CTA CTC CTA TT-3′; 6) 5′-CCC CAT AAT AGA CTG TGA CCC ACA AGC TAG CCG GGG CTG TCG GGG CTG T-3′; 7) 5′-CAC AGC CCC GAC AGC CCC GGC TAG CTT GTG GGT CAC AGT CTA TTA TGG GG-3′; 8) 5′-ATC GGC GCC CGG GGG ACA CCT GAC TGT TAC TTC AGC CAC-3′; and 9) 5′-ATC GGC GCC ACC CAG GAC TGC TCC TTC CAA CAC AGC CCC-3′. For mFlex:gp140, primers 1 and 2 in PCR 1, and primers 3 and 4 in PCR 2, were used; for mFlex:gp120, primers 1 and 2 for PCR 1, and primers 3 and 5 for PCR 2 were used; for tPA-mFlex:gp120, primers 8 and 3 for PCR 1, and primers 3 and 5 for PCR 2 were used; for tPA-hFLex:gp140, primers 9 and 6 for PCR 1, and primers 7 and 4 for PCR 2 were used; and for tPA-hFLex:gp120, primers 9 and 6 for PCR 1, and primers 7 and 5 for PCR 2 were used.

We performed three separate PCR to construct individual chimeric genes as described previously (43, 44). In PCR 1, amplification of the *FLex* gene was done and PCR 2 was performed to amplify $HIV\text{-}1_{(89.6p)}$ *env* (gp120, gp140) sequences (44). PCR 3 (fusion PCR) used the first primer in PCR 1 and the second primer in PCR 2 (e.g., for mFLex:gp140, primers 1 and 4) to sew fragments together. DNA sequencing was performed to ascertain the authenticity of clones. All these genes were cloned into the vaccine vector pNGVL-7, using appropriate cloning sites described for each gene construct.

Transfection and protein Western blot

293T cells (1.5×10^6) were transfected with 10–15 μg each of the DNA vaccine constructs using the calcium phosphate method (Invitrogen, San Diego, CA). The proteins were probed with an anti-$HIV_{89.6P}$ Env Ab (raised in rabbits; a gift of Dr. R. Doms (University of Pennsylvania, Philadelphia, PA)) or biotinylated anti-human Flt-3L Ab (0.2 μg/ml; R&D Systems, Minneapolis, MN). To detect proteins using the ECL Western blot detection system (44, 45), we used secondary Abs, either goat anti-rabbit IgG H+L-HRP conjugate (Geno-Tech, St. Louis, MO; 1:5000) or NeutrAvidin-HRP conjugate (1/10,000 dilution; Pierce, Rockford, IL).

Sucrose density gradient centrifugation

The oligomeric state of gp120 and the FLex:gp120 fusion proteins were investigated using sucrose density gradient centrifugation. The proteins in the transfected 293T cell supernatants were concentrated ~50-fold using Centricon 100 filters (Amicon, Beverly, MA). Approximately 100 μl of the concentrated supernatants was loaded onto 10-ml 10–25% continuous sucrose gradients, which were centrifuged in an SW41 rotor (Beckman Coulter, Fullerton, CA) for 20 h at 40,000 rpm at 4°C. Fractions of 1.0 ml were collected manually. Five microliters from each fraction was analyzed on nonreducing and reducing SDS-PAGE as described previously for Western blot.

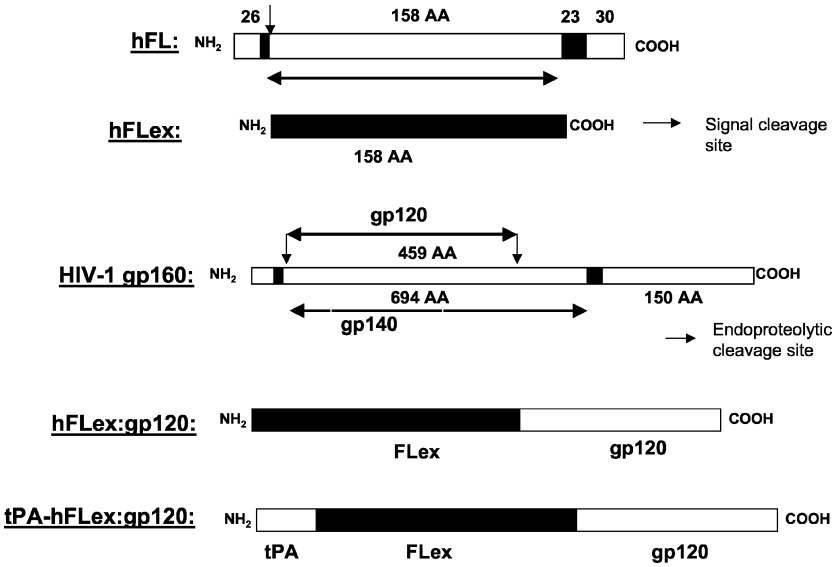

FIGURE 1. Schematics of Flt-3 ligand, HIV-1 $gp160_{89.6P}$, and FLex:HIV envelope fusion proteins. We made gene constructs to produce various proteins in mammalian cells in vitro and to use them as DNA vaccines in mice, using the PCR methodology described in *Materials and Methods*. To make fusion proteins, gene fragments were amplified and ligated together. For example, the gene encoding the extracellular domain (158 aa for human and 165 aa for mouse) of Flt-3L was linked to the gene coding for HIV-1 $gp120_{89.6P}$ (459 aa) or $gp140_{89.6P}$ (694 aa). Both gp120 and gp140 were derived from HIV-1 $160_{89.6P}$ (42). The former lacks extracellular gp41 transmembrane and cytoplasmic domains, whereas the latter lacks the transmembrane and cytoplasmic domains of gp160. The resulting chimeric genes were sequenced and estimated to have M_r of ~140 kDa for both hFLex:gp120 and mFLex:gp120. For the fusions containing gp140 as a partner with mFLex or hFLex, the expected molecular mass is ~160 kDa. In constructs, we used the tPA signal sequence for efficient ER translocation in place of the gp120 signal sequence (50). The genes encoding tPA-hFLex:gp120, tPA-mFLex:gp120, and tPA-gp120 were used as DNA vaccines in the present study.

In vivo DC expansion by plasmid DNAs

Mice were injected with plasmids expressing hFLex:gp120, mFLex:gp120, hFLex, or gp120 as per the protocol described (46). Briefly, 10 μg of DNA was diluted in 1.6 ml of saline and injected into mice through their tail vein over a period of 10 s using a 27.5-gauge needle. At day 7 postinjection, spleens were collected and made into a single-cell suspension. Following this, RBC were lysed, and cells were counted and surface stained for CD11b, CD11c, CD3, and B220, after incubating with Fc block Ab (2.4G2). The Abs used were the following: CD11b-allophycocyanin (M1/70), CD11c-PE (HL3), CD3-PerCP (145-2C11), and B220 PerCP (RA3-6B2) (BD PharMingen, San Diego, CA). We used PerCP for both CD3 and B220 so as to exclude these populations while doing the analysis for DCs. The surface staining was conducted on ice for 20 min in 100 μl of FACS buffer (Dulbecco's PBS containing 1% BSA plus 0.2% sodium azide). The cells were then washed twice with FACS buffer and then fixed with 200 μl of 1% PFA. The cells were acquired using FACSCalibur (BD Biosciences, Mountain View, CA) and analyzed on FlowJo 3.4 (Tree Star, San Carlos, CA).

Mice and immunizations

Female BALB/c (6–8 wk old) were obtained from Charles River Breeding Laboratories (Wilmington, MA) via the National Cancer Institute, Frederick Cancer Facility, and maintained in the vivarium at the Yerkes National Primate Research Center (Atlanta, GA) according to the Institutional Animal Care and Use Committee guidelines. Age-matched mice were used in all of our DNA vaccination experiments. In DNA vaccination experiments involving in vivo DC expansion, mice were injected i.v. with 10 μg of hFLex DNA encoding native FLex protein 3 days before intramuscular (i.m.) delivery of DNA vaccine constructs or control plasmid DNA. Correspondingly, for vaccination without DC expansion, the same amount of the vector DNA, pNGVL-7, was injected i.v. (no DC expansion). i.m. injections of DNA vaccines were delivered into each of the tibialis anterior muscles of a mouse at a concentration of 1 μg/μl (total DNA, 100 μg/mouse) in 50 μl of phosphate buffer (pH 7.4). For boost responses, we used the same route and amounts of DNA that were used for the primary response on day 97 post-primary immunization. A final i.m. boost was given with 100 μg of gp120 DNA on day 128 after the first boost. Blood was drawn from the retro-orbital plexus on days 14, 28, and 90 for primary response, and on days 80 and 114 after the first boost and day 12 after the second boost for PBMCs and serum collection.

Preparation of mouse PBMCs and intracellular cytokine staining (ICC) assay

Blood was collected through the orbital plexus into tubes containing heparin (5 U/ml) in PBS. Histopaque (1 ml at 37°C) was under-layered and spun at 2000 rpm (20°C) for 20 min without applying the brakes. The interphase containing PBMCs was collected and washed twice with PBS and once with complete medium (RPMI 1640 containing 10% FCS). We followed a protocol described by Murali-Krishna et al. (47) for ICC assay. Briefly, PBMCs (10^5–10^6 cells/well) were stimulated in vitro (96-well U-bottom plates) in the presence of gp120 V3 loop peptide (0.1 μg/ml; *IGPGRAFYAR*, restricted by *H2-D*^{d} in BALB/c mice) (48, 49) and its absence or an irrelevant peptide (lymphocytic choriomeningitis virus, NP118) in 10% RPMI 1640 complete medium with brefeldin A (Golgiplug; BD PharMingen). After stimulation for 6 h, the cells were washed once with FACS buffer (Dulbecco's PBS containing 1% BSA plus 0.2% sodium azide) and then stained with FITC-conjugated anti-CD3 (clone 17A2; BD PharMingen) and PerCP-labeled anti-CD8 (clone 53-6.7) Abs each at a concentration of 1 μg/10^6 cells for 20 min on ice in 100 μl of buffer. The cells were then washed twice with FACS buffer, permeabilized, and stained for intracellular IFN-γ with the Cytofix/Cytoperm staining kit (BD PharMingen) according to the manufacturer's recommendations. For detection of CD69 on CD8$^+$ T cells, we used PE-conjugated rat anti-mouse Ab (clone H1.2F3; BD PharMingen) and for IFN-γ, allophycocyanin-labeled rat anti-mouse IFN-γ (clone XMG1.2; BD PharMingen). The cells were then washed twice, resuspended in 200 μl of 1% PFA, and acquired on a FACSCalibur within 24 h, and data analysis was performed using FlowJo 3.4 software.

ELISA

Standard ELISA was used to probe a number of biochemical and immunological properties of FLex:gp120 fusion proteins: 1) quantitation, 2) ability to bind hCD4, 3) measurement of Ab titers, and 4) IgG subclass determination. We used Maxisorb (Nunc, Naperville, IL) ELISA plates. For each of the procedures, the following primary and secondary Abs and substrates were used. For quantitative assay, monoclonal anti-Flt-3L Ab (R&D Systems), rhFlt-3 (R&D Systems), biotinylated anti-human Flt-3L Ab (0.2 μg/ml), and NeutrAvidin-HRP conjugate were used. For hCD4 binding assay, 293T cells (10^5) were transfected with the vectors expressing hCD4 (43), gp120, hFLex:gp120, and mFLex:gp120. Detergent lysates of the hCD4-transfected cells were diluted in bicarbonate buffer before coating the ELISA plates. The medium supernatants collected from the transfected cells (gp120, hFLex:gp120, and mFLex:gp120) were added to the ELISA plates and incubated for 1 h after a blocking step. Specific CD4 binding was assessed using the anti-HIV$_{89.6P}$ Env Ab, goat anti-rabbit IgG-HRP conjugate, and *o*-phenylenediamine (OPD). For Ab titers in capture ELISA, sheep anti-gp120 Ab (50 μg/ml; Cliniqa, Fallbrook, CA) and anti-mouse IgG coupled to biotin (1:2000; Sigma-Aldrich, St. Louis, MO) were used. To do this assay, the plates were coated with sheep anti-gp120 Ab in bicarbonate buffer. After a blocking step (5% milk powder in PBS-Tween), HIV-1 gp140$_{89.6P}$ expressed from recombinant vaccinia virus (a gift from Dr. R. Doms) was added to the plates for 1 h at 37°C. After three washes, sera collected from the control and immunized mice were serially diluted in PBS-Tween. After a 2-h incubation at 37°C, the anti-mouse IgG coupled to biotin (1:2000) was added first, followed by NeutrAvidin-HRP to the plates, which were then developed using OPD. The reactions were quenched in 50 μl of 2 N sulfuric acid, and absorbance was measured at 492 and 650 nm. The reciprocal of the serum dilution showing an OD reading greater than the OD of vector control sera (0.1) was taken as the ELISA Ab titer. For IgG subclass determination, rat anti-mouse IgG1a or rat anti-mouse IgG2a coupled to biotin (Southern Biotechnology Associates, Birmingham, AL) were used. The substrates were 1-step-Turbo TMB-ELISA (Pierce) for the quantitative assay and OPD (0.5 mg/ml; in 0.1 M citric acid, 0.2 M $NHPO_4$, and 0.1% H_2O_2) for the rest of the ELISA. All procedures were followed according to the manufacturer's instructions.

Statistical analysis

In all animal experiments, we used five mice per group, from which mean and SD were calculated. For Ab titers, we used pooled sera from five mice. The significance of differences between groups in gp120-specific CD8$^+$ T cells was determined by Student's *t* test. A series of *t* tests (between hFLex:gp120 and gp120 in the presence and absence of DC expansion) were performed to determine whether the observed differences in the outcome demonstrated true differences in the populations or were the result of random sampling error. A value of $p < 0.05$ was considered significant.

Results

Expression of DNA vaccines encoding the HIV-1 envelope glycoproteins (gp120$_{89.6P}$ and gp140$_{89.6P}$) fused to mFLex and hFLex

The chimeric gene constructs were transfected into 293T cells, and their expression was analyzed in both lysates and medium supernatants of the transfected cells. We used an anti-HIV$_{89.6P}$ Env Ab that recognized the expressed proteins on Western blots, and the molecular mass of each of the fusion proteins was consistent with the expected size with respect to the number of amino acids and glycosylation (Fig. 2). However, intracellular gp120 was heterogeneous in nature, reflecting perhaps differential glycosylation (24 *N*-linked glycosylation sites) of the protein in the endoplasmic reticulum (ER). In contrast, gp120 in the medium represents a highly mature homogenous protein having the glycosylation sites modified in the Golgi complex before it is released from the cell (Fig. 2*C*). Furthermore, the fusion proteins comprised of gp140 and hFLex or mFLex were made as single protein bands reflecting their different folding characteristics and glycosylation in the ER, but in the medium fraction, they show some heterogeneity due to a mixture of cleaved (Flex:gp120) and uncleaved proteins (Flex:gp140). Importantly, the fusion proteins tPA-hFLex:gp120 and tPA-mFLex:gp120, having the tPA signal, were expressed and secreted efficiently in 293T cells (Fig. 2*A*, *lanes 5* and *6*). This is consistent with previous findings that the tPA signal efficiently transfers proteins from the cytosol to the ER membrane or lumen (50). Note that we loaded only 1/50 of the amount of each of the fusion proteins with tPA signal sequence on the gel for Western detection (Fig. 2*B*, *lanes 5* and *6*). However, tPA:hFLex:gp140 was not efficiently made or secreted and showed a pattern of expression very

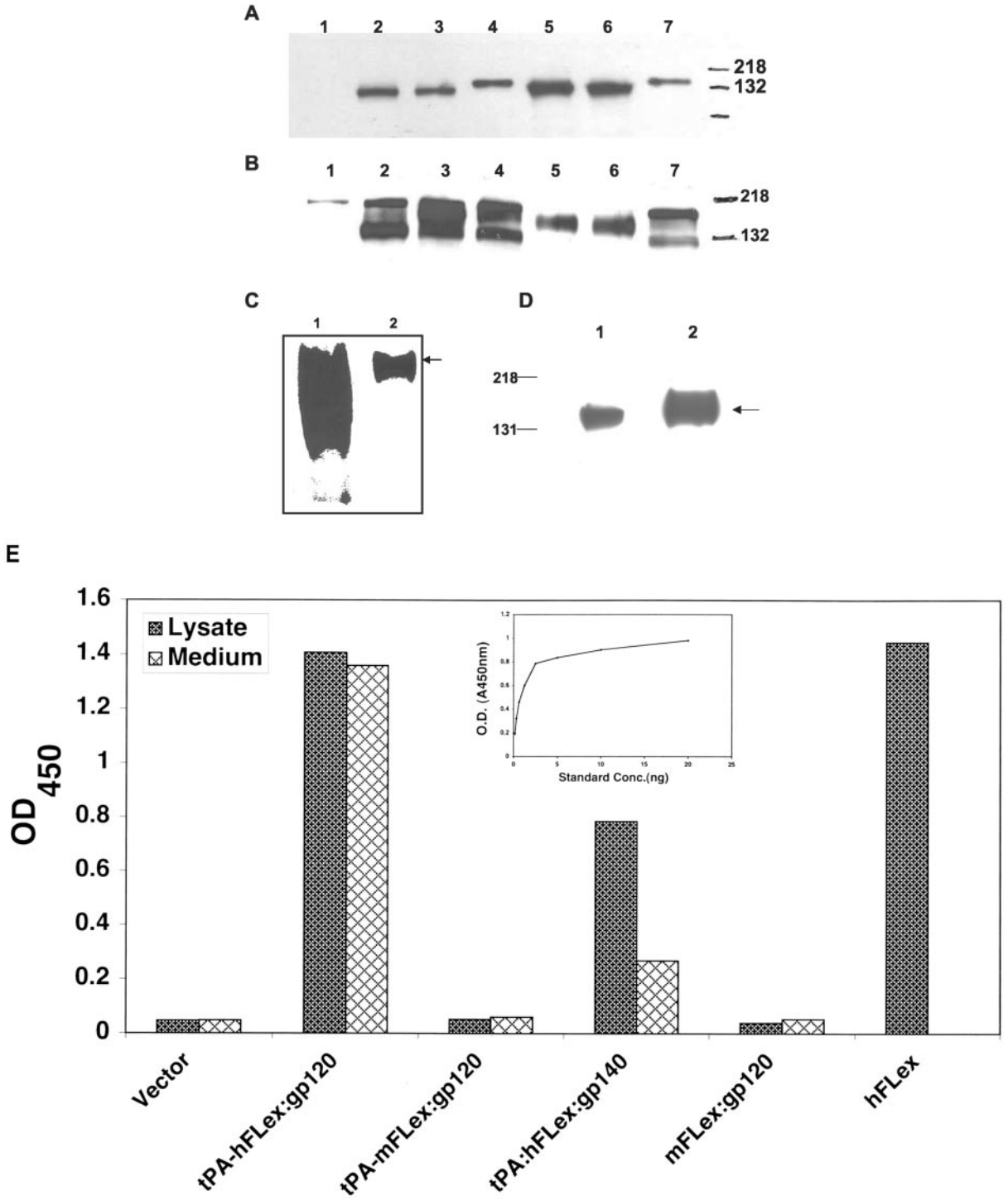

FIGURE 2. Western blot analysis of HIV-1 $gp120_{89.6P}$ and FLex:$gp120_{89.6P}$ fusion proteins. Transfection and protein analysis were done as described in *Materials and Methods*. *A* and *B*, The proteins were detected in cell lysates representing intracellular (*A*) and medium supernatants representing extracellular (*B*) fractions. *Lane 1*, pNGVL-7; *2*, gp140; *3*, mFLex:gp120; *4*, mFLex:gp140; *5*, tPA-mFLex:gp120; *6*, tPA-hFLex:gp120; and *7*, tPA-hFLex:gp140. The proteins in *lanes 5* and *6* represent only 1/50 of the amount loaded in other lanes. *C*, gp120 loaded in cell lysate (*lane 1*) and medium supernatant (*lane 2*). *D*, The fusion protein tPA-hFLex:gp120 detected by the Env Ab (*A* and *B*, *lane 6*) was also probed with anti-rhFLex Ab in both cell lysate (*lane 1*) and medium supernatant (*lane 2*) fractions. Arrows in *C* and *D* indicate the positions of gp120 and tPA-hFLex:gp120 secreted into the medium. Note that intracellular gp120 runs as a ladder on the gel representing differential glycosylation in the ER. *E*, Quantitation of FLex:gp120 fusion proteins. The cell lysate (1/100 dilutions) and medium supernatant fraction (1/500 dilutions) of transfected cells with tPA-hFLex:gp120 and tPA-hFLex:gp140 were quantified by ELISA using monoclonal rhFLex Ab. To determine specificity of the anti-rhFLex Ab, tPA-mFLex:gp120 was included in the assay and this Ab, as expected, did not react with mFLex protein. rhFLex protein (0.15–20 ng) served as standard in ELISA to quantify the expressed fusion proteins.

similar to that of gp140, mFLex:gp120, or mFLex:gp140 (those having their own gp120 signal). The properties of some of the fusion proteins have thus suggested that the efficiency of ER translocation could also depend on the nature of the proteins. The lysate and medium fractions of tPA-hFLex:gp120 were also recognized by a species-specific (human) anti-Flt-3L Ab on Western blots

(Fig. 2*D*, *lanes 1* and *2*). Therefore, we used the same Ab to quantitate the amount of secreted tPA-hFLex:gp120 in the medium by ELISA, and it was found to be 4 μg/ml (Fig. 2*E*). Although both tPA-hFLex:gp120 and tPA-mFLex:gp120 were made at equivalent amounts as assessed by Western analysis (Fig. 2, *A* and *B*, *lanes 5* and *6*), the anti-human Flt-3L Ab, as expected, did not recognize tPA-mFLex:gp120 on ELISA. In the current study, we use the genes for hFLex:gp120 and mFLex:gp120 and gp120 (expressed using the tPA signal sequence) to test their efficacy as DNA vaccines in the induction of $CD8^+$ T cells and Ab immune responses in mice. Before this vaccine testing, we conducted experiments to glean information on the biochemical and biological properties of the fusion proteins using in vitro and in vivo assays (Figs. 3 and 4).

Oligomeric status of gp120 and the fusion proteins hFLex:gp120 and mFLex:gp120

HIV-1 gp120 has been shown to exist predominantly as a monomer, but recent data suggest that it could form oligomeric complexes in the intracellular compartments of the cell (51–54). Any modification to gp120 could potentially alter the assembly status in the cell. Sucrose gradient analysis was done to assess the subunit composition of gp120 as well as the fusion proteins hFLex:gp120 and mFLex:gp120. HIV-1 gp120 had sedimented at or near the bottom of the gradient with a small fraction sedimenting at a position that is consistent with it being a monomer (Fig. 3*A*). Addition of a reducing agent (2-ME) in the gradient produced gp120 that was predominantly in the monomeric state with some form in the dimeric position on the gradient (Fig. 3*B*). The gradient analysis demonstrated that HIV-1 gp120 formed aggregates, which could be reduced to its unit monomeric structure.

Interestingly, analysis of hFLex:gp120 and mFLex:gp120 revealed some unique sedimentation behavior. The hFLex:gp120 protein, although larger in size compared with gp120, sedimented predominantly as monomers or dimers with a small fraction of the protein sedimenting at the bottom of the gradient as an aggregate (Fig. 3*C*). In contrast, mFLex:gp120 showed a dramatic shift in the sedimentation profile and appears to be largely monomeric with fractions of the protein sedimenting as both dimers and aggregates on the gradient (Fig. 3*E*). The reducing agent had completely converted both the fusion proteins to their unit monomeric structure (Fig. 3, *D* and *F*). Thus, the addition of hFLex or mFLex to HIV-1 gp120 appears to have profoundly altered the subunit composition of the fusion proteins, which now form monomeric or dimeric structures, instead of forming oligomers or aggregates as in the case of their parental gp120 (Fig. 3). Flt-3L has been shown to exist predominantly as a dimer (55, 56), and therefore, it is likely that sequence determinants in FLex could influence subunit composition of hFLex:gp120 and mFLex:gp120 in mammalian cells.

hFLex:gp120 and mFLex:gp120 bind hCD4 and are biologically active in inducing DC expansion in vivo

As shown in Fig. 3, the fusion proteins assembled as oligomeric complexes. Therefore, we wanted to examine their ability to bind hCD4, a major HIV-1 receptor. To this end, we performed ELISA, and the supernatant medium fractions of transfected cells (gp120, mFLex:gp120, and hFLex:gp120) were assessed to bind hCD4 coated onto ELISA plates (Fig. 4*A*). All three glycoproteins bind CD4 with an equal efficiency suggesting that addition of FLex sequences to gp120 did not affect the ability of gp120 to bind hCD4. The FLex protein expressed from a plasmid DNA was able

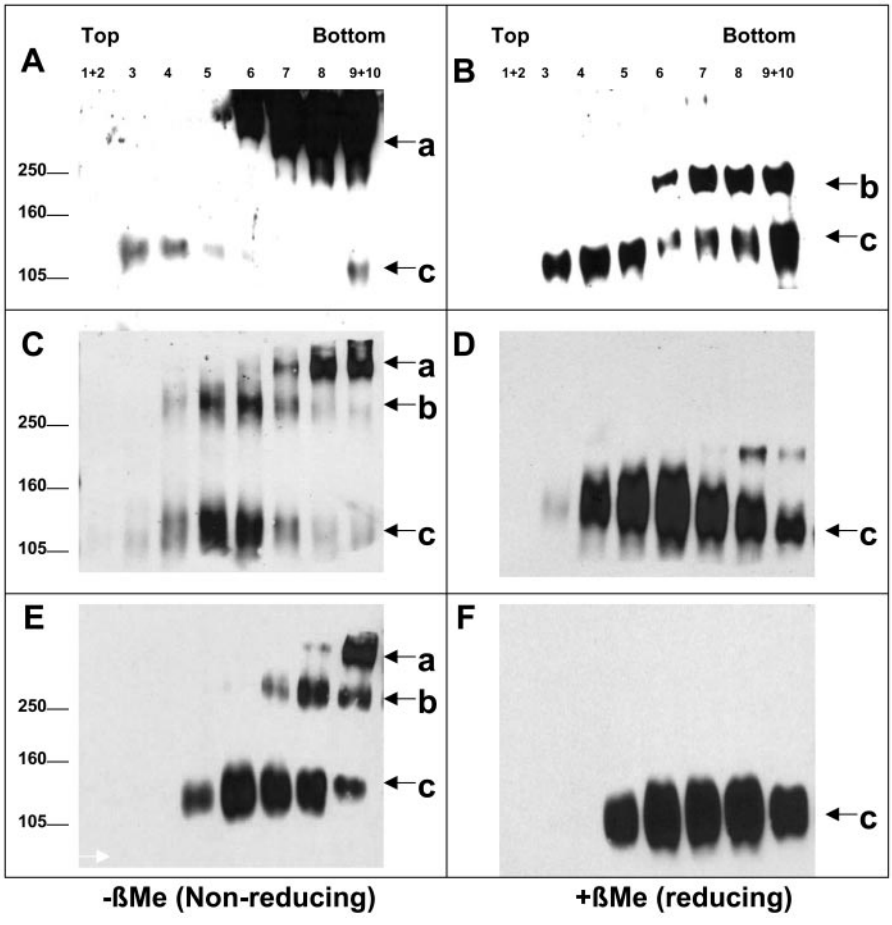

FIGURE 3. Subunit characterization of the secreted FLex:gp120 fusion proteins. 293T cells were transfected with plasmid vectors expressing gp120 (*A* and *B*), tPA-hFLex:gp120 (*C* and *D*), and tPA mFLex:gp120 (*E* and *F*). The concentrated medium was loaded on top of 10–25% sucrose gradients and ultracentrifuged. One-milliliter fractions were collected from the top of the gradient. Fractions 1 and 10 represent, respectively, the top and bottom of the gradient. Five microliters from each fraction was dissolved in either nonreducing ($-\beta$Me) (*A*, *C*, and *E*) or reducing ($+\beta$Me) (*B*, *D*, and *F*) buffer and analyzed by Western blotting. Arrows indicate oligomer (*a*), dimer (*b*), and monomer (*c*).

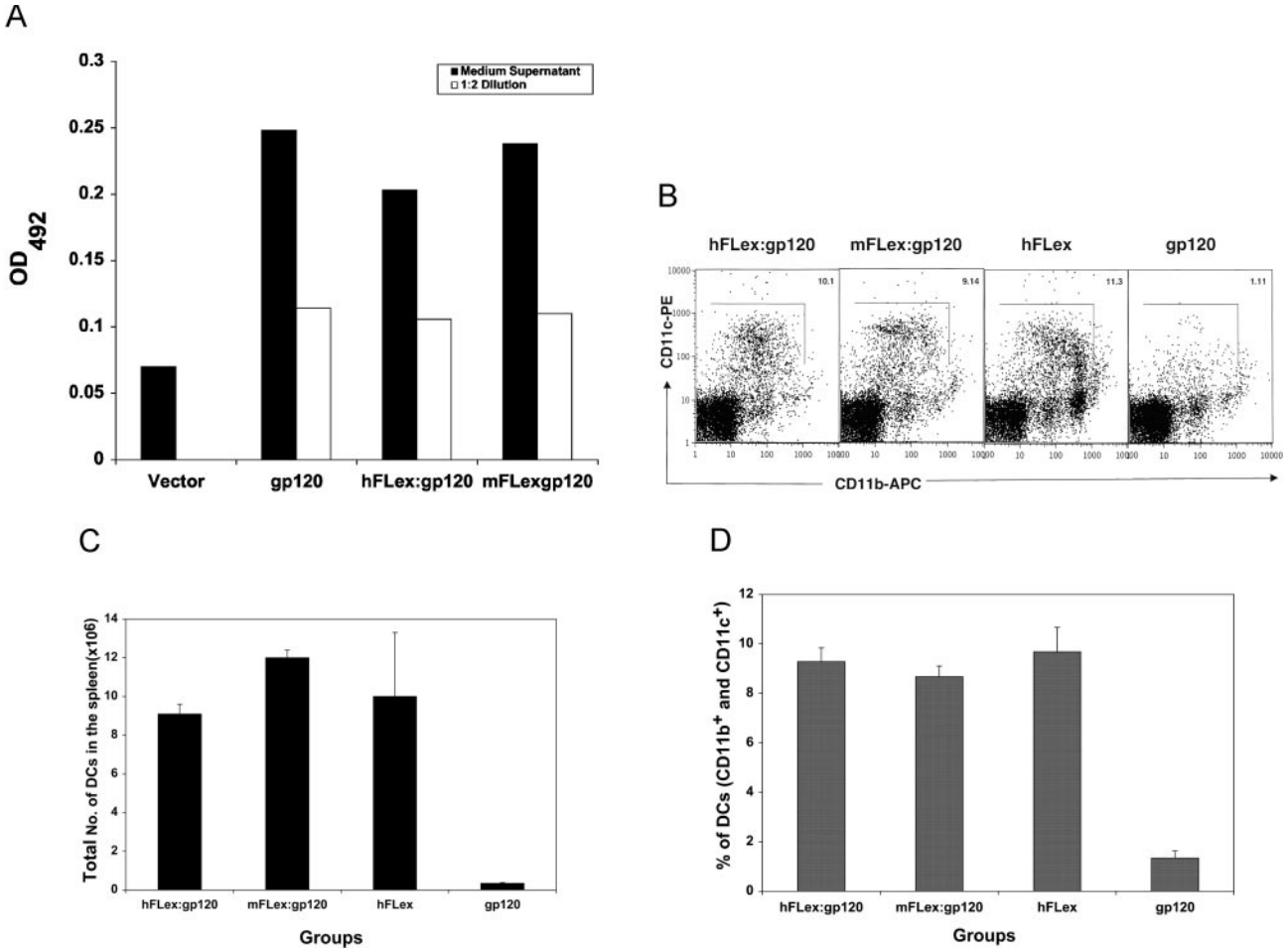

FIGURE 4. The fusion proteins bind to CD4 in vitro and induce DC expansion in vivo. *A*, The medium supernatants of hFLex:gp120, mFLex:gp120, and gp120 were used to bind hCD4 on ELISA. The plates were developed with anti-$HIV_{89.6P}$ Env Ab and goat anti-rabbit IgG-HRP conjugate and developed with OPD as described in *Materials and Methods*. gp120 and the fusion proteins hFLex:gp120 and mFLex:gp120 bind hCD4 to a similar extent. *B*, Mice (three per group) were injected with DNA constructs expressing gp120, hFLex, hFLex:gp120, and mFLex:gp120, and spleens were isolated on day 7 postinjection. Splenocytes (1×10^6 from each mouse) were surface stained with CD8-FITC, CD11C-PE, CD11b-allophycocyanin, CD3-PerCP, and B220-PerCP, and gated on CD11c- and CD11b-positive cells as described in *Materials and Methods*. Representative data from one mouse from each group are presented in *B*. *C* and *D*, The mean absolute numbers and percentage of DC from three mice per group are represented (one of two repeat) in the histogram with SD values.

to induce a dramatic expansion in the numbers of the DC population in mice (46). To test whether the FLex portion in the fusion proteins, hFLex:gp120 and mFLex:gp120, could retain Flt-3L activity, we chose four groups of mice (three mice per group) and injected them with DNA vaccine constructs and FLex DNA. At day 7 postinjection, we observed an increase (2-fold) in spleen size and cellularity in mice injected with plasmid DNA expressing either hFLex:gp120 or mFLex:gp120, but naive mice and those injected with gp120 DNA, as expected, did not show such increases (data not shown). Total spleen cells were analyzed for the expression of cell surface markers, CD11b and CD11c, in a population that does not express CD3 and B220 (nonlymphoid and non-B cell populations). This population of cells represents both myeloid and lymphoid subsets of DC (Fig. 4*B*). As shown in Fig. 4, *B–D*, with the exception of mice injected with gp120 DNA, all three groups of mice showed increases in the number of $CD11b^+CD11c^+$ DC in their spleen. This property of the fusion proteins illustrates dramatically that the FLex portions (human and mouse forms) in hFLex:gp120 and mFLex:gp120 are biologically active in inducing the expansion of DC (by 9- to 10-fold), and this expansion is comparable to that induced by hFLex DNA expressing the nonfusion, native hFLex protein (Fig. 4, *C* and *D*). Taken together, these results have clearly demonstrated that physical linkage of FLex to gp120 did not affect the functional property of either of the protein components in the fusion proteins (CD4 binding in the case of gp120 and DC expansion in the case of FLex).

Induction and maintenance of gp120-specific $CD8^+$ T cells in mice immunized with DNA vaccines expressing gp120, hFLex: gp120, and mFLex:gp120

We tested the ability of DNA vaccines expressing the fusion proteins hFLex:gp120 and mFLex:gp120 to induce Ag-specific $CD8^+$ T cell immune responses in mice. We chose eight groups of mice for this set of experiments as shown in the immunization protocol (Fig. 5). i.v. injection of mice with FLex DNA induced DC expansion, which was discernable from day 2 (data not shown), and therefore, day 3 post-i.v. injection was determined to be appropriate to analyze the efficacy of DNA vaccination under conditions of DC expansion in vivo. To test this hypothesis, four groups of mice (1–4) were injected with FLex DNA and were then immunized i.m. (100 μg/mouse, both primary and booster injections) with DNA vaccine constructs expressing gp120, hFLex:gp120, and mFLex:gp120, or the empty vector at day 3 post-i.v. injection (referred to as day 0 of DNA vaccination). For the other four groups

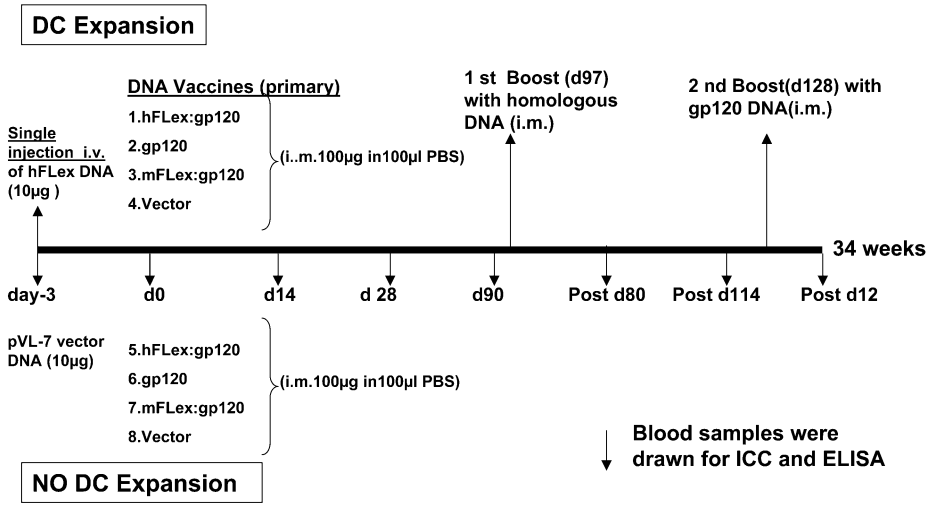

FIGURE 5. DNA vaccination protocol for analysis of HIV-1 gp120-specific immune responses in mice.

(5–8), the same protocol was followed except that the mice in these groups were injected with empty vector DNA before DNA vaccination, i.e., no FLex DNA-induced in vivo DC expansion. We sampled blood at the primary and booster phases of the immune response (sampling done during primary at days 14, 28, and 90, and for booster at days 80 and 114 after the first boost) (Fig. 5). PBMCs were stimulated with gp120 peptide to enumerate gp120-specific $CD8^+$ T cells in immunized mice. This protocol was also designed to elucidate CD8 memory responses that could persist in mice for a longer period of time.

In the mouse groups (1–4) where DC were expanded by FLex DNA injection, hFLex:gp120 DNA induced significantly higher frequencies of gp120-specific $CD8^+$ T cells compared with those induced by gp120 DNA or mFLex:gp120 DNA at day 14 of the primary phase of the immune response. Both gp120 DNA and mFLex:gp120 DNA induced very similar levels of $CD8^+$ T cells at this time. At day 90 after the first i.m. injection of the DNA vaccines, the frequency of *env*-specific $CD8^+$ T cells was reduced to low or undetectable levels (Fig. 6, *A* and *B*). This kinetics of T cell activation appears to be consistent with the generation and subsequent evolution of Ag-specific $CD8^+$ T cells in response to acute viral infections (47, 57–60). DNA vaccination appears to induce higher frequencies of gp120-specific $CD8^+$ T cells at day 14 (priming/activation phase of the immune response), and this number wanes in the absence of antigenic stimuli for 3 mo (90 days). This represents the contraction phase in the immune response, perhaps very similar to that observed in viral infections.

At day 97, a booster i.m. injection was given to these groups of mice; we analyzed gp120-specific $CD8^+$ T cells in PBMC of the control and immunized mice at day 80 after the first boost. Mice that were primed with hFLex:gp120 DNA harbored significantly high levels of gp120-specific $CD8^+$ T cells at day 80 (20-fold increase compared with the numbers at day 90 after priming) after receiving a single booster injection of the same DNA vaccine. This level of gp120-specific $CD8^+$ T cells persisted for a long time even after day 114 postboost (Fig. 6, *A* and *B*). In contrast, mice that received gp120 DNA or mFLex:gp120 DNA had shown only modest increases (6- to 7-fold) in the frequency of gp120-specific $CD8^+$ T cells at both days 80 and 114 after a booster injection with the corresponding DNA vaccine constructs. However, mice that were immunized (prime boost) with mFLex:gp120 DNA showed a relatively better response compared with mice immunized with gp120 DNA alone. These studies have shown that vaccination of mice with hFLex:gp120 DNA induced quantitatively very superior response, followed in the order of magnitude by mFLex:gp120 DNA and gp120 DNA under the same conditions of DC expansion in vivo.

A similar kinetic profile was observed in the generation and evolution of gp120-specific $CD8^+$ T cells in mice immunized with DNA vaccines expressing gp120, hFLex:gp120, and mFLex:gp120 in the absence of FLex DNA-induced DC expansion (Fig. 6, *C* and *D*). Importantly, the magnitude of both primary and booster responses was not significantly altered in the case of gp120 DNA and mFLex:gp120 DNA. However, mice immunized with hFLex:gp120 DNA have been shown to harbor lower levels (1.5 to 2-fold) of gp120-specific $CD8^+$ T cells compared with that induced by the same DNA vaccine in the presence of DC expansion. This level of *env*-specific $CD8^+$ T cells was significantly higher than that achieved either by the vaccine construct expressing mFLex:gp120 or gp120. As expected, control groups, irrelevant peptide (IRR control), or isotype controls did not elicit any gp120-specific $CD8^+$ T cells confirming the specificity of vaccine-induced immune responses in mice (Fig. 6*E*). Importantly, gp120-specific memory $CD8^+$ T cells persisted for a long time (day 114 postboost) in all vaccinated mice, i.e., 206 days after receiving a primary i.m. injection of the corresponding DNA vaccine. These experiments have revealed unique features of gp120 DNA vaccines that were modified to carry the extracellular portion of Flt-3L, a growth factor for DC in vivo. The FLex:gp120 DNA vaccines have consistently induced significantly (20-fold) higher frequencies of *env*-specific $CD8^+$ T cells compared with that induced by gp120 DNA both in the presence and absence of DC expansion. Additionally, it is intriguing that hFLex:gp120 DNA elicits much (2- to 3-fold) higher $CD8^+$ T cell immune responses than mFLex:gp120 DNA, a vaccine that expresses mFLex in the context of the fusion protein.

Differential induction of gp120-specific Ab responses by DNA vaccines expressing gp120, hFLex:gp120, and mFLex:gp120

To detect gp120-specific Ab titers, we collected sera from the same set of mice used for the analysis of gp120-specific $CD8^+$ T cells at the primary and booster phases of the immune response in the presence and absence of DC expansion. Unlike in the case of the $CD8^+$ T cell experiments, all the mice were boosted additionally by i.m. route with gp120 DNA (100 μg/100 μl each). Mice immunized with DNA vaccines expressing gp120 and hFLex:gp120 contained only low levels of gp120-specific Abs after two booster

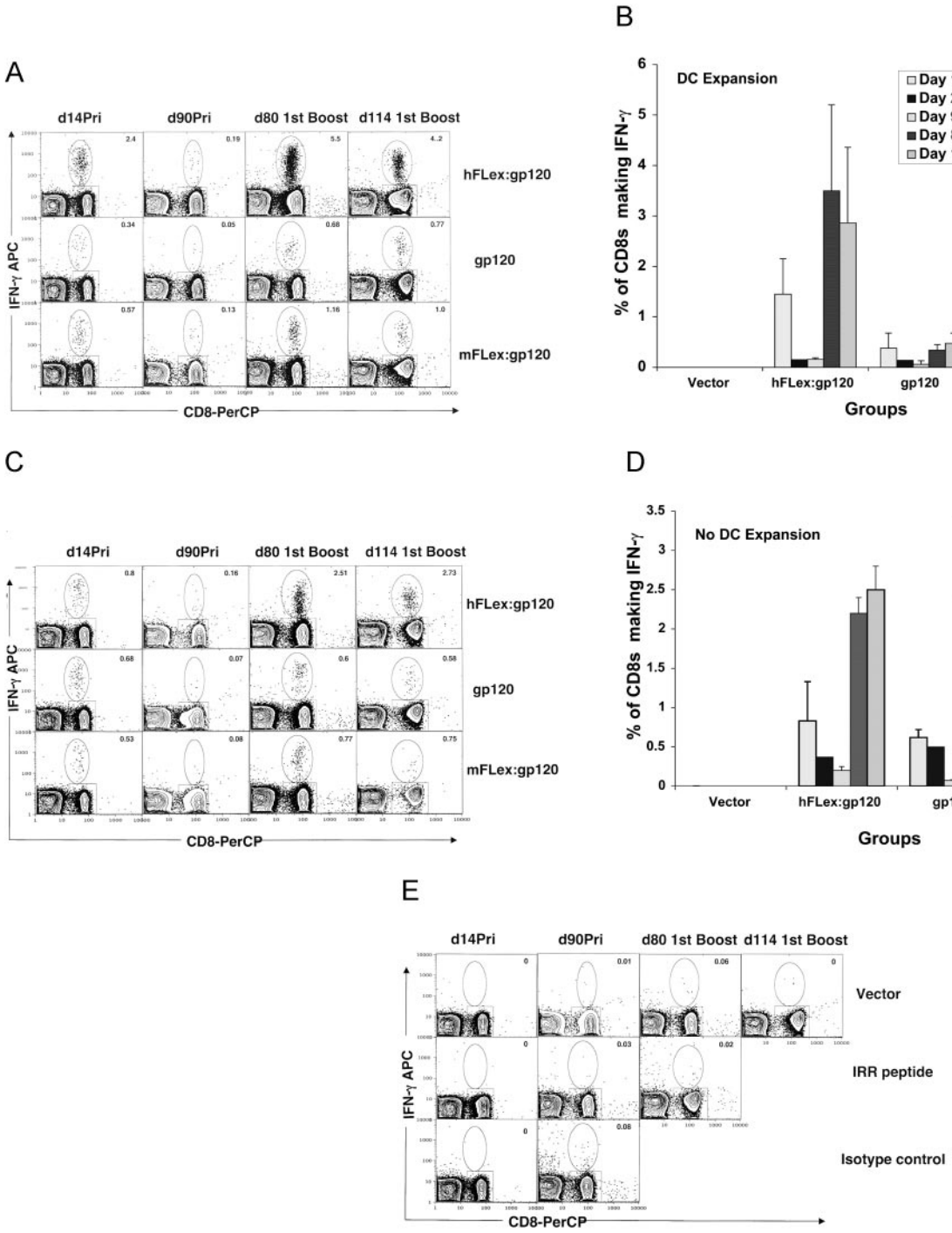

FIGURE 6. Generation and evolution of gp120-specific $CD8^+$ T cells in mice immunized with DNA vaccines in the presence and absence of DC expansion. To perform these experiments, groups of mice were injected i.v. with either FLex DNA (DC expansion) (*A*) or the empty vector pNGVL-7 (no DC expansion) (*C*) 3 days before DNA vaccination. As DNA vaccines, the gene constructs expressing gp120, hFLex:gp120, and mFLex:gp120 were injected i.m. into mice (*A* and *C*) at day 3 postinjection as enumerated in Fig. 5. Primary and booster immunizations were performed as described in *Materials and Methods*. PBMC from the control and immunized mice were isolated at days 14, 28, and 90 for the primary response or at days 80 and 114, respectively, for the boost and memory responses after a booster injection. $CD8^+$ T cells expressing intracellular IFN-γ in response to the gp120 V3 loop peptide (ICC) were identified using appropriate Abs in a FACSCalibur. The frequency of gp120-specific $CD8^+$ T cells in one representative mouse in a group of five per immunization is shown. As controls, PBMC from the vector pNGVL-7-immunized mice, irrelevant peptide, and isotype controls are included (*E*). The average frequency of gp120-specific $CD8^+$ T cells from five mice of each group at primary and booster immunizations is shown in *B* (DC expanded group) and in *D* (no DC expansion). The data are represented in the histograms with SD values. Representative data from one experiment (of two repeat) are presented.

injections, whereas mFLex:gp120 DNA elicited a robust Ab response (64-fold) after first boost injection in the absence of DC expansion (Fig. 7). However, the pattern of the Ab response was different in mice that were induced to have more DC before DNA vaccination (Fig. 7*A*). In the case of gp120 DNA and hFLex:gp120 DNA, the first booster injection induced 2- to 4-fold higher levels

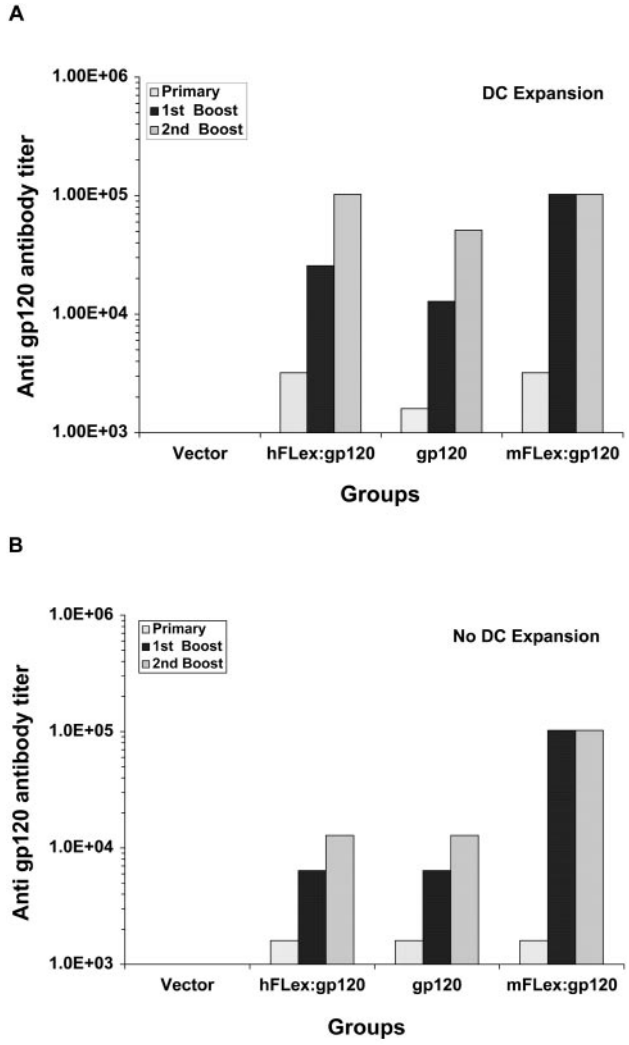

FIGURE 7. Induction of anti-gp120 Abs in the presence and absence of DC expansion. Sera were collected from the same two sets of mouse groups (DC expanded (*A*) and no DC expansion (*B*); see Fig. 6) at day 14 for primary, and days 80 and 12 after the first and second booster injections, respectively. Ab titers were quantified using capture ELISA as described in *Materials and Methods*. The gp120-specific Ab titers were from the pooled sera of five mice in the same group. Representative data from one experiment (of two repeat) are presented.

of gp120-specific Abs, respectively, compared with the levels induced in the absence of DC expansion. Interestingly, mice immunized with mFLex:gp120 DNA showed very similar patterns (significantly higher levels) of Ab induction in the absence and presence of DC expansion in the first and second booster injections (Fig. 7, *A* and *B*). A second booster with hFLex:gp120 DNA had resulted in the levels of Abs comparable to that induced by mFLex:gp120 DNA in the first or second booster injection. However, gp120 DNA showed a booster response that is higher (2- to 4-fold) than that induced by the same DNA vaccine in the absence of DC expansion, but is lower than that induced by mFLex:gp120 or hFLex:gp120. Also the subclass typing of the Ab response induced by both human and mouse Flex:gp120 DNA vaccines showed a mixed immune response of IgG1 and IgG2a (Th1- and Th2-dependent immune responses, data not shown). The primed and boosted sera of mice injected with hFLex:gp120 were tested for hFLex Abs by Western blotting analysis and resulted in the absence of anti-hFLex Abs (data not shown). Thus, these experiments have revealed a number of intriguing aspects of Ab responses induced by DNA vaccines expressing gp120, mFLex:gp120, and hFLex:gp120.

Discussion

In the present study, we have provided evidence that HIV-1 gp120 linked to FLex elicited higher frequencies of gp120-specific $CD8^+$ T cells and Ab responses. In the case of mice immunized with a DNA vaccine expressing hFLex:gp120, gp120-specific $CD8^+$ T cells persisted for an extended period of time at high levels as CD8 memory cells. Although similar levels of expression were achieved for all gene constructs (mFLex:gp120 and hFLex:gp120) in vitro, it is intriguing that only hFLex:gp120 DNA was capable of eliciting a robust $CD8^+$ T cell response. In contrast, mFLex:gp120 DNA was superior in inducing gp120-specific Ab response even after the first booster of DNA vaccination. Although Gp120 DNA was poorly immunogenic in inducing Ag-specific $CD8^+$ T cells, it was capable of inducing high levels of anti-gp120 Abs in second boost immunization in the presence of DC expansion.

The mechanisms by which mFLex:gp120 and hFLex:gp120 behave differently in the elicitation of immune responses are not clearly understood. It is likely that each of the proteins was able to target a distinct population of DC subsets that are capable of priming one or the other type of immune response (24, 26, 31). How this is achieved is only a matter of speculation. However, a recent report suggests that hFLex and mFLex, when introduced as recombinant proteins, induce a distinct set of DC populations in mice (30). The hFLex protein induced both $CD4^-CD8^+$ and $CD4^-CD8^-$ DC subsets to a similar extent, whereas mFLex was shown to preferentially affect the numbers of $CD4^-CD8^+$ subsets in mice. This study had revealed for the first time functional differences between recombinant hFLex and mFLex proteins in their ability to expand DC subsets, which could have significant influence in the induction of T cell and Ab immune responses in mice. However, it is interesting to note that both hFLex:gp120 and mFLex:gp120 proteins were able to induce $CD11b^+CD11c^+$ and $CD11b^{low}CD11c^+$ DC subsets at comparable levels. This DC expansion was monitored in mice that were injected (i.v.) with the plasmids expressing hFLex:gp120 and mFLex:gp120.

Ag-specific immune responses (CD8 and Ab) were measured in i.m. vaccination of mice with all the gene constructs in the presence and absence of DC expansion induced by hFLex DNA. Although there was a measurable difference (2-fold) in the frequency of Ag-specific $CD8^+$ T cells in the presence of DC expansion in vivo, this difference is not directly correlated with the number of DC present (10-fold) at the time of vaccination. It is not clear whether hFLex:gp120 DNA or mFLex:gp120 DNA could induce local DC expansion at the site of DNA injection (muscle cells) to elicit robust immune responses. Even though the receptor for Flt-3L has been restricted to hemopoietic stem cells (HSC), recent studies have revealed that myocytes harbored bone marrow-derived HSC (61, 62). It is not clear what function these cells could perform in myocytes. HSC in muscle cell could potentially have a role in signaling mechanisms mediated by the FLex proteins (63). This notion awaits further experimentation as to how these mechanisms could modulate DNA vaccine-induced immune responses.

In this study, we have performed immunization experiments that delivered DNA fusion vaccines through the i.m. route. Hung et al. (64) have shown that a Flt-3L:E7 DNA fusion vaccine delivered to the epidermis of mice by gene gun was capable of eliciting robust E7-specific $CD8^+$ T cells with greater efficacy in reducing tumor burden. Although both modes of DNA delivery appear to elicit Ag-specific $CD8^+$ T cell responses, the mechanism of immune induction would probably be different in either case in terms of the nature of Ag and participating cell types as DNA vaccine targets

both tissue compartments. We have used a naturally secreted protein, gp120, which is a poor immunogen, to generate a fusion protein consisting of FLex and gp120. The FLex:gp120 fusion proteins were efficiently secreted into the medium, and this was important to induce Ab responses. However, hFLex:gp120 failed to elicit significant levels of Abs in the absence of DC expansion, whereas mFLex:gp120 was very efficient in eliciting this arm of the immune response in both the presence and absence of DC expansion. This strongly suggests that secretion per se is not sufficient for induction of Ab responses, and other regulatory mechanisms appear to be operative in in vivo elicitation of DNA vaccine-induced immune responses. Even though DNA vaccines have been shown to target DC directly for production of Ag for presentation through the endogenous MHC class I pathway, DC are capable of acquiring Ag from neighboring cells (cross-presentation) that undergo necrosis or programmed cell death (5–6). Hung et al. (64) used a nuclear/cytoplasmic protein, E7, of human papillomavirus-16 to make a fusion protein with hFLex directing E7 to the secretory pathway, and the fusion protein Flt-3L:E7 was able to elicit high frequencies of E7-specific $CD8^+$ T cells with no or little enhancement in the levels of $CD4^+$ T cells. Although both of our studies used different model Ags to assess the potency of FLex-based DNA vaccines, they have revealed remarkably common features of this vaccine approach in inducing a robust $CD8^+$ T cell response in mice. Interestingly, physical linkage of Ags to other cellular proteins has immune-enhancing effects when tested as DNA vaccines in mice (65, 66)

A recent report has elucidated the roles of cytokines and hemopoietic growth factor, Flt-3L, in modulating DNA vaccine-induced immune responses in mice (37). After three injections of DNA vaccines in the absence or presence of the gene(s) encoding one or more of cytokines, or Flt-3L, the authors have shown a complex pattern in the induction of either $CD8^+$ T cell or Ab response (37). The differential effects on immune responses by these immunomodulatory factors appear to be dependent on the nature of Ag (HIV-1 Nef vs Env). In this study, we used a short-term culture system to enumerate gp120-specific $CD8^+$ T cells in PBMC of mice during the primary and booster phases of the immune response elicited by DNA vaccines expressing gp120, hFLex:gp120, and mFlex:gp120. At the single-cell level, our study did not reveal any enhancement in the frequency of gp120-specific $CD8^+$ T cells in mice immunized with gp120 DNA in the presence and absence of DC expansion in vivo. These assays have proven to accurately measure T cell immune responses elicited by viral infections (47, 67) or by the various modes of DNA vaccination (68, 69). This could be due to a number of factors. Both studies used different experimental protocols in terms of timing and duration of booster immunizations as well as the mode of Ag delivery with or without other immune-modulatory factors.

Moore et al. (37) used very frequent immunization regimens involving three i.m. injections followed by a bulk assay to measure the levels of cytokines (IFN-γ) in supernatants of spleen cells in the presence of relevant proteins or peptides. We injected mice i.m. only once before giving a booster injection at day 97 (a long rest period). We have shown clearly the persistence of $CD8^+$ T cells in mice immunized in all the vaccine constructs, but mice immunized with FLex:gp120 DNA consistently harbored higher frequencies of gp120-specific $CD8^+$ T cells after a booster injection compared with that induced by gp120 DNA. However, it is not clear whether the protocol of Moore et al. (37) induced any substantial levels of $CD8^+$ T cells capable of persisting for a long period of time. Repeated immunizations could negatively impact on the outcome of the memory immune response (57). Such immunization regimens could drive the effector cells into the death pathway reducing their number and ultimately the size of the memory pool. Understanding the relationship between various aspects of the effector and memory phases of DNA vaccine-induced immune responses would be of great importance in the design of effective HIV/AIDS vaccines.

Our studies have demonstrated a clear potential for this novel FLex-based DNA vaccine approach to be applicable in other animal model systems. However, it is conceivable that the recombinant gene could have the ability for integration into the host chromosome, limiting its use in humans as DNA vaccines. It is very encouraging that recombinant FLex protein when tested in people did not elicit any overt toxicity (18). Modification of protein Ags having a functional FLex protein (like FLex:gp120) attached to them would provide a novel means of Ag delivery for inducing immune responses in animals. Such approaches would have to minimize potential integration problems inherent in DNA vaccines that express cellular proteins as part of vaccine adjuvants. It is interesting to note that DNA vaccines delivered with IL-2 Ig (a cellular cytokine protein) DNA achieved great potency in eliciting HIV-specific $CD8^+$ T cell responses in rhesus macaques (70).

The mechanisms by which FLex:gp120 DNA induces high levels of both $CD8^+$ T cell and Ab responses are not clearly defined. The poor immunogenicity of HIV envelope protein gp120 could be partly due to its inherent biological properties (71, 72, 73). Covalent attachment of FLex to gp120 could have converted gp120 to a better immunogen for induction of robust immune responses in mice. Additionally, gp120 appears to exist predominantly as monomers or aggregates in in vitro tissue culture systems, and if this property were retained in vivo, it could serve as a very poor substrate for recognition by immune cells. In contrast, the Flex:gp120 fusion proteins appear to be more soluble, exhibiting dimeric or monomeric forms in vitro. These proteins are functionally active in the induction of DC expansion in vivo, and therefore, they are likely to assume stable structures that are as native as possible so they would be recognized by immune cells for elicitation of both $CD8^+$ T cell and Ab responses. Thus, our studies have revealed that vaccine strategies could involve modifications of Ags acquiring novel functional properties in in vivo vaccination settings. However, this approach of DNA vaccination should also minimize any potential problems inherent in some of the vaccine strategies (Refs. 64, 65, and 70, and this study). Since these approaches have used cellular genes for immune enhancement, integration of these genes into the host chromosome is a distinct possibility. However, other modes of vaccination (viral vectors, protein delivery) could incorporate the fusion proteins of this sort for both priming and booster immunizations in animals and in humans.

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