An HIV-1 clade C DNA prime, NYVAC boost vaccine regimen induces reliable, polyfunctional, and long-lasting T cell responses

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The EuroVacc 02 phase I trial has evaluated the safety and immunogenicity of a prime-boost regimen comprising recombinant DNA and the poxvirus vector NYVAC, both expressing a common immunogen consisting of Env, Gag, Pol, and Nef polypeptide domain from human immunodeficiency virus (HIV)-1 clade C isolate, CN54. 40 volunteers were randomized to receive DNA C or nothing on day 0 and at week 4, followed by NYVAC C at weeks 20 and 24. The primary immunogenicity endpoints were measured at weeks 26 and 28 by the quantification of T cell responses using the interferon γ enzyme-linked immunospot assay. Our results indicate that the DNA C plus NYVAC C vaccine regimen was highly immunogenic, as indicated by the detection of T cell responses in 90% of vaccinees and was superior to responses induced by NYVAC C alone (33% of responders). The vaccine-induced T cell responses were (a) vigorous in the case of the env response (mean 480 spot-forming units/10^6 mononuclear cells at weeks 26/28), (b) polyfunctional for both CD4 and CD8 T cell responses, (c) broad (the average number of epitopes was 4.2 per responder), and (d) durable (T cell responses were present in 70% of vaccinees at week 72). The vaccine-induced T cell responses were strongest and most frequently directed against Env (91% of vaccines), but smaller responses against Gag-Pol-Nef were also observed in 48% of vaccinees. These results support the development of the poxvirus platform in the HIV vaccine field and the further clinical development of the DNA C plus NYVAC C vaccine regimen.

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Abbreviations used: EV02, EuroVacc 02; GPN, GagPol-Nef; ICS, intracellular cytokine staining; MVA, modified vaccinia virus Ankara; SEB, staphylococcal enterotoxin B; SFU, spot-forming unit(s); SIV, simian immunodeficiency virus.

The control of HIV transmission remains one of the most pressing public health priorities for the 21st century. All past experience suggests that a vaccine will be the only intervention able to control the HIV epidemic. Although many successful antiretroviral drugs have been developed with enormous impact on HIV-associated morbidity and mortality, access to antiretroviral therapy remains limited to only 5% of the total population of HIV-infected subjects

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in developing countries (1). Even in the face of open access to therapy, HIV transmission continues.

Over the past 10 yr there has been a progressive increase in resources for vaccine research, allowing the generation of several candidate vaccines capable of stimulating anti-HIV immunity (2–4). The ideal HIV vaccine should be safe, thermostable, able to elicit both humoral (antibody) and cellular (both CD4 and CD8 T cells) effector functions, and to induce durable protective immunity (2–5). The goal of the "antibody" vaccines is to induce neutralizing antibodies to provide immunity that prevents or limits infection; so far, these have been recombinant envelope protein vaccines (2–4). One envelope protein vaccine has been tested in a phase III efficacy clinical trial, without any protective effect (2–4). This candidate, as well as other tested envelope protein vaccine candidates, induces antibodies with neutralizing activity against laboratory-adapted strains of HIV-1, but these antibodies are largely ineffective against primary HIV-1 isolates. Strategies to develop high titres of neutralizing antibodies with broad activity against primary HIV-1 isolates remain elusive.

In contrast, relatively significant advances have been made in the development of "T cell" vaccine candidates. T cell vaccines are unlikely to prevent infection but may control HIV replication after infection, leading to attenuation of HIV disease (6, 7). The scientific rationale for developing T cell vaccines is based on several observations, including: (a) the presence of vigorous CD4 and CD8 HIV-1–specific T cell responses in HIV-1–infected subjects that are long-term nonprogressors (8); (b) the in vivo observation in the simian immunodeficiency virus (SIV) model of AIDS in macaques that the depletion of CD8 T cells is associated with rapid loss of control of virus replication (9); and (c) the recent observation that CD4 and CD8 T cell responses endowed with multiple functional capacities, particularly IL-2 secretion and proliferation in addition to typical effector functions (cytotoxic activity and secretion of IFN-γ, TNF-α, and MIP-1β), are associated with better control of virus replication (10–12).

Most T cell vaccines use virus vectors to induce T cell immunity, particularly adenovirus and poxvirus vectors (3, 4, 13). Adenovirus vectors used alone or in combination with plasmid DNA-based vaccines have been shown to induce vigorous T cell responses (14). However, the high seroprevalence to adenoviruses in target populations remains a major issue for adenovirus vectors, even though recent data seem to indicate that vector immunity may be circumvented by higher vaccine doses and/or combination with DNA-based vaccines (3, 4, 6, 13). Vector immunity seems to be a lesser problem for poxvirus vector–based T cell vaccines, due to the diminishing prevalence of a vaccinia-experienced population.

The efficiency of poxvirus vectors in eliciting T cell responses has been variable (15). Limited immunogenicity (17% response) has been shown in a study using recombinant DNA in combination with a modified vaccinia virus Ankara (MVA) expressing Gag protein (consensus of HIV-1 clade A) and several immunodominant CD8 T cell epitopes (16). A more recent study using the same vaccines at a higher dose showed enhanced immunogenicity (17). However, the response rate based on ex vivo functional analysis (IFN-γ ELISPOT and proliferation) remained limited at a 40% response, and the T cell responses induced were exclusively due to CD4 T cells. Similarly, the ex vivo immunogenicity of a canarypox candidate vaccine, ALVAC, currently being tested in combination with an Env protein vaccine in a phase III clinical trial, was in the range of 30% (18). This work describes the immunogenicity of a prime-boost regimen composed of recombinant DNA and the poxvirus vector NYVAC, both expressing common immunogens consisting of Env, Gag, Pol, and Nef proteins of the HIV-1 clade C isolate CN54. We performed a comprehensive functional analysis of the vaccine-induced T cell responses, including quantification of T cell responses by IFN-γ ELISPOT and functional characterization of CD4 and CD8 T cell responses (IFN-γ, IL-2, and TNF-α secretion, proliferation, and degranulation activity) by polychromatic flow cytometry and epitope mapping. Our results indicate that

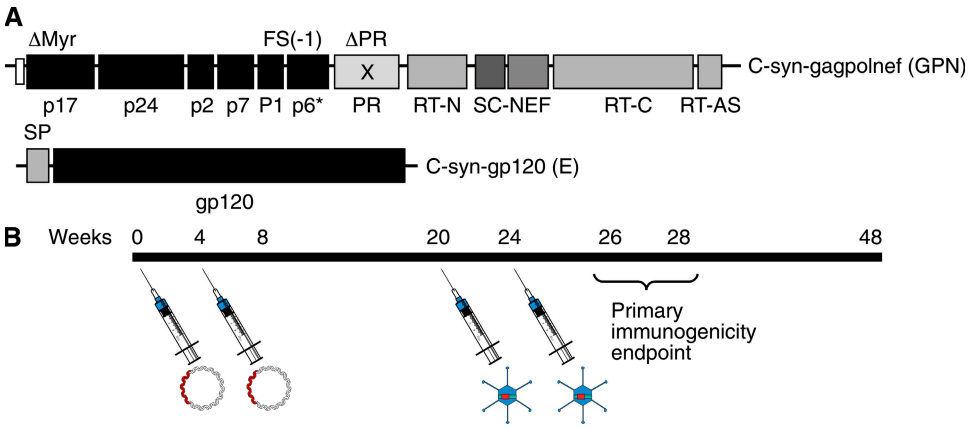

Figure 1. RNA- and codon-optimized GPN and Env gene vector inserts. (A and B) Schematic representation of EV02 study design. ΔMyr, myristoylation-deficient; FS (-1), placing Gag and PolNef in one reading frame by removing the natural frameshift; ΔPR, protease-inactivated; RT-N, RT-C NH2 terminal and C-terminal part of the HIV reverse transcription; SC-Nef, scrambled Nef; RT-AS, active site of RT; SP, signal peptide.

the DNA/NYVAC vaccine combination induced ex vivo T cell responses in 90% of immunized volunteers and that these responses were vigorous, polyfunctional, broad, and durable.

RESULTS

Study design

The main objectives of the EuroVacc 02 (EV02) trial were to evaluate the safety and immunogenicity of the prime-boost regimen, DNA C plus NYVAC C, compared with NYVAC C alone. The DNA and the poxvirus vector NYVAC both expressed fused Gag-Pol-Nef and the gp120 subunit of Env of the HIV-1 clade C isolate, CN54 (Fig. 1 A). The design was open for participants and clinical investigators, without a placebo control, and 40 volunteers were randomized to receive DNA C or nothing on day 0 and at week 4, followed by NYVAC C at weeks 20 and 24 (Fig. 1 B). The participants received 2 × 2-ml injections of DNA C (1.05 mg per ml and a total dose of 4.2 mg) intramuscularly in the right and left vastus lateralis and a 1-ml injection of NYVAC C ($10^{7.7}$ $CCID_{50}$ per ml) intramuscularly in the deltoid. The primary immunogenicity endpoints were measured at weeks 26 and 28 by the quantification of T cell responses using the IFN-γ ELISPOT assay. The T cell responses were also measured on day 0 and at weeks 5, 20, 24, and 48. Comprehensive analyses of the demographics of the trial population and of the safety of the vaccine regimens have been described (unpublished data and reference 19). The results indicated that both DNA C and NYVAC C candidate vaccines are safe and well tolerated.

Proportion of responders after vaccination with DNA C plus NYVAC C compared with NYVAC C alone

Although 40 subjects entered the EV02 study, only 35 had completed vaccination. 20 subjects were randomized to the DNA C plus NYVAC C group and 15 to the NYVAC C–alone group. As mentioned above, T cell responses were measured at different time points during the 48-wk study using the IFN-γ ELISPOT assay. The primary immunogenicity endpoints were, however, evaluated on the basis of the proportion of subjects with positive vaccine-induced T cell responses at weeks 26 and 28. There was a clear and significant difference (P = 0.003) in the proportion of subjects with positive vaccine–induced T cell responses within the two study groups. The proportion of responders was 90% (18 out of 20) in the DNA C plus NYVAC C group compared with 40% (6 out of 15) in the NYVAC C–alone group (Fig. 2 A). Based on an intention-to-treat analysis of all 40 participants (assuming that missing equals no response) 83% (19 out of 23) in the DNA C plus NYVAC C group responded compared with 35% (6 out of 17) in the NYVAC C–alone group (difference 47%; 95% confidence interval 20–75%; P = 0.0034). It is important to note that one of the six responders in the NYVAC C–alone group had a detectable response (in the range of 200 spot-forming units [SFU]/10^6 cells) not only at weeks 26 and 28, but also at weeks 5 and 20 before vaccination. Therefore, although this subject had to be considered positive at weeks 26 and 28, the T cell response observed was clearly nonspecific and was not considered for analyses. On the basis of the above considerations, the proportion of subjects with vaccine-induced specific T cell responses was 33% (5 out of 15) in the group vaccinated with NYVAC C alone. The assessment of vaccine-induced T cell responses at different time points has indicated that the proportion of responders after the DNA C vaccination was low, for instance 10% at week 5 after two vaccinations and 35% at week 20 before NYVAC C boost (Fig. 2 B). Furthermore, the proportion of responders in the DNA C plus NYVAC C group mostly peaked (17 out of 20) at week 24, 4 wk after the first NYVAC C boost, and the proportion of responders was still 80% at week 48, 6 mo after the completion of the vaccination (Fig. 1 B). Only two subjects within the NYVAC C–alone group maintained positive vaccine–induced T cell responses at week 48 (Fig. 2 B).

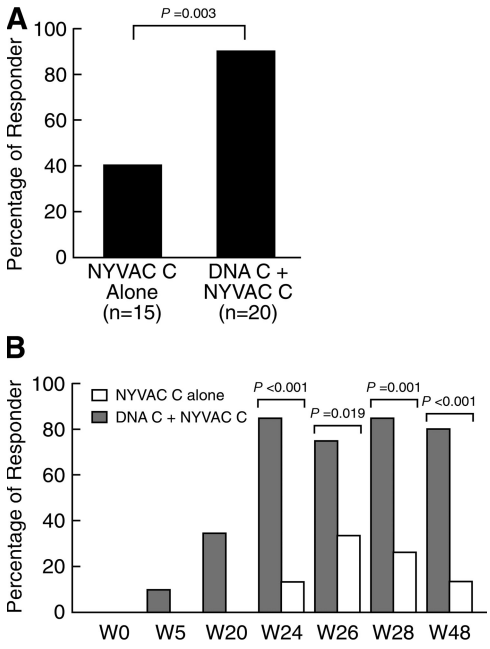

Figure 2. Immunogenicity of DNA C plus NYVAC C versus NYVAC C–alone vaccine regimens. (A) Percentage of responders in the two study groups at weeks 26/28, i.e., primary endpoints of the study. The percentage of responders was calculated on the basis of volunteers with a positive IFN-γ ELISPOT assay at weeks 26/28. (B) Percentage of responders at the different time points across the duration of the study.

Magnitude of vaccine-induced T cell responses

Vaccine-induced T cell responses were assessed using the IFN-γ ELISPOT assay after the stimulation of blood mononuclear cells with a panel of 464 peptides (15 mers overlapping by 11 amino acids) grouped in eight pools (50–60 peptides per pool). The peptides encompassed the Env, Gag, Pol, and Nef proteins of HIV-1 and were designed based on the sequence of the immunogens expressed by the DNA and NYVAC that were derived from the CN54 clade C isolate. The magnitude and the distribution of vaccine-induced T cell responses against Env, Gag, Pol, and Nef HIV-1 proteins in all the responders within the two study groups are shown in Fig. 3 A.

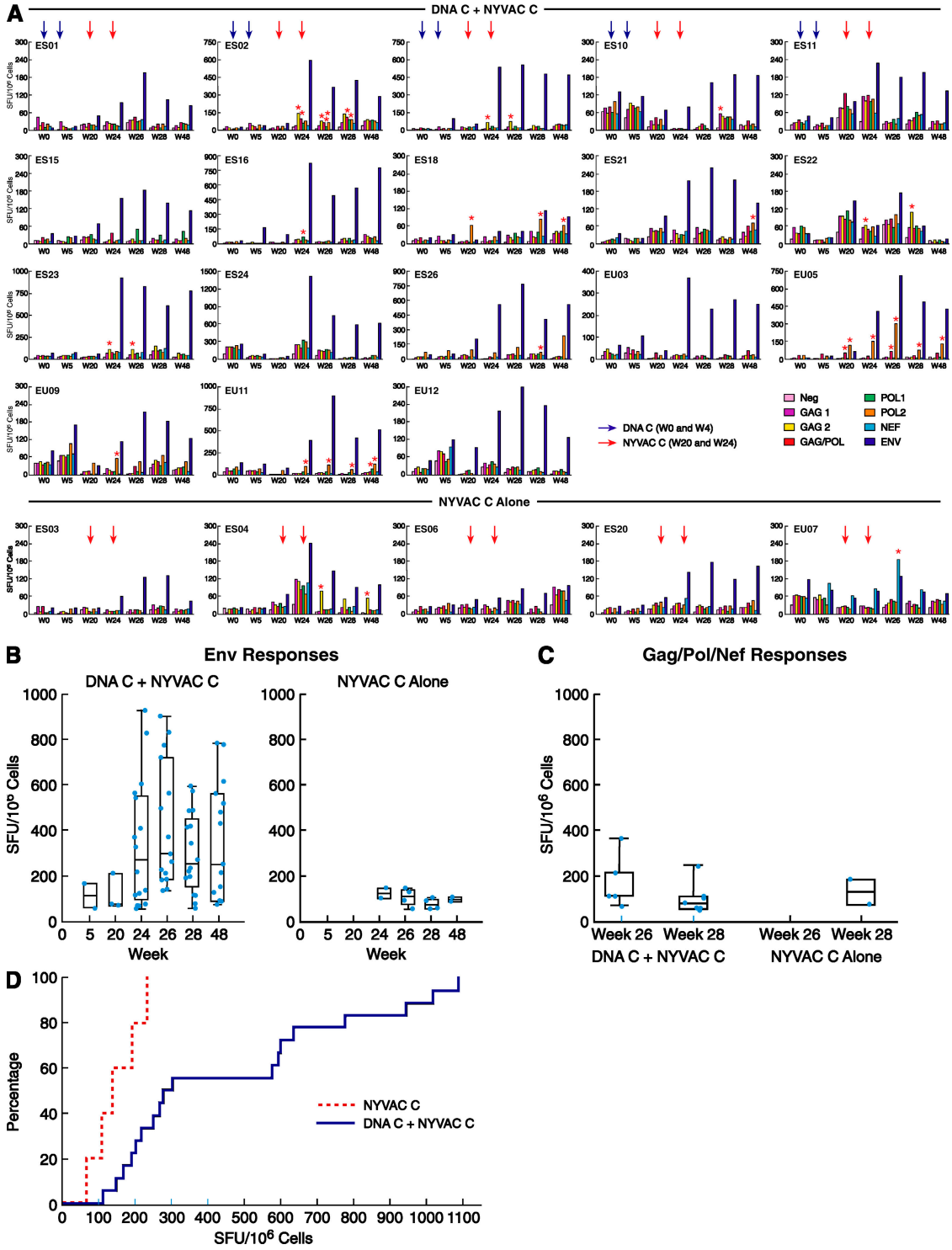

Figure 3. Magnitude of the vaccine-induced T cell responses. (A) Individual patterns of the T cell responses as measured by the frequencies of IFN-γ–secreting cells against different peptide pools encompassing the Env, Gag, Pol, and Nef proteins in an ELISPOT assay are shown for all responders in both study groups. Each bar corresponds to the reactivity against a different peptide pool, and positive responses (against Gag, Pol, and Nef) are indicated by a star. The Env-specific responses correspond to the sum of the mean of the responses induced after stimulation with the two Env peptide pools. (B) Median magnitude of vaccine-induced T cell responses against Env at different time points across the duration of the study. (C) Median magnitude of Gag-, Pol-, and Nef-specific vaccine-induced T cell responses at weeks 26 and 28. (D) Cumulative distribution of the sum of SFU/10^6 cells of positive responses at week 26 or 28 by randomization group. For each participant, the week of maximum T cell response (sum SFU) was chosen. Each step corresponds to the maximal IFN-γ ELISPOT value for each participant.

Vaccine-induced T cell responses were predominantly directed against Env in the DNA C plus NYVAC C and NYVAC C–alone groups. At weeks 26/28 (primary endpoints), Env-specific responses were observed in 21 out of 23 responders (91%) in both groups, whereas Gag, Pol, and Nef vaccine–induced T cell responses were observed in 11 out of 23 of volunteers (48%). Transient Gag- and Pol-specific T cell responses were found in two additional volunteers (ES16 and EU09) at week 24, and Nef-specific T cell responses were only found in one volunteer (EU07) in the NYVAC C–alone group (Fig. 3 A). The responses against Gag, Pol, and Nef were generally transient and substantially lower in magnitude compared with the Env-specific responses.

At week 26, the median (mean ± SD) Env-specific IFN-γ–secreting T cells was 299 (480 ± 339) SFU/10^6 cells within the study group vaccinated with DNA C plus NYVAC C compared with 131 (139 ± 69) SFU/10^6 cells within the group vaccinated with NYVAC C alone (Fig. 3 B). The differences in the magnitude of T cell response between the two groups were significant (P = 0.013). At week 28, the numbers were 246 (324 ± 196) SFU/10^6 cells for the DNA C plus NYVAC C and 76 (78 ± 24) SFU/10^6 cells for the NYVAC C–alone group (P = 0.005) (Fig. 3 B). The majority (>80%) of Gag-, Pol-, and Nef-specific responses were only detected at weeks 26 and 28 in the DNA C plus NYVAC C group and were not measurable at week 48 (Fig. 3 C and not depicted). These responses were measurable only at week 26 in the NYVAC C–alone group (Fig. 3 C). The median magnitude of Gag-, Pol-, and Nef-specific T cell responses was ~100 SFU/10^6 cells (Fig. 3 C).

In Fig. 3 D, one observes the individual maximum responses at week 26 or 28 (each "step" corresponds to the maximum response measured in each responder) and the percentage of participants per arm with a maximum response up to a certain value. In the NYVAC C–alone group, the maximum response was just above 200 SFU/10^6 cells, and 40% had a maximum response of 100 or fewer. In the DNA C plus NYVAC C group, the maximum response was almost 1,100 SFU/10^6 cells, and 33% had a maximum response of >600 SFU/10^6 cells.

Distribution of the vaccine-induced T cell responses in CD4 and CD8 T cell populations

The distribution of vaccine-induced T cell responses in CD4 and CD8 T cell populations was assessed in 3 out of 5 responders of the NYVAC C–alone group and in 16 out of 18 responders of the DNA C plus NYVAC C group. Only volunteers with IFN-γ ELISPOT responses in the range of 100 SFU/10^6 blood mononuclear cells or above were characterized using polychromatic flow cytometry. The vaccine-induced T cell responses were mediated by CD4 T cells in all the investigated 19 responders (3 in the NYVAC alone and 16 in the DNA C plus NYVAC C groups). However, vaccine-induced CD8 T cell responses were observed in addition to CD4 T cell responses in 1 out of 3 responders in the NYVAC C–alone group and in the 8 out of 16 (50%) responders in the DNA C plus NYVAC C group (not depicted). Representative flow cytometry profiles of Env-specific IFN-γ–secreting CD4 and CD8 T cells in six out of the seven responders with both CD4 and CD8 T cell responses vaccinated with DNA C plus NYVAC C are shown in Fig. 4 A. The magnitude of vaccine-induced CD4 and CD8 T cell responses was either similar or higher in CD8 T cells (Fig. 4 A). The characterization of vaccine-induced CD4 and CD8 T cell responses was performed mostly for Env-specific responses as well as for the Gag-specific responses when the magnitude was >100 SFU/10^6 cells.

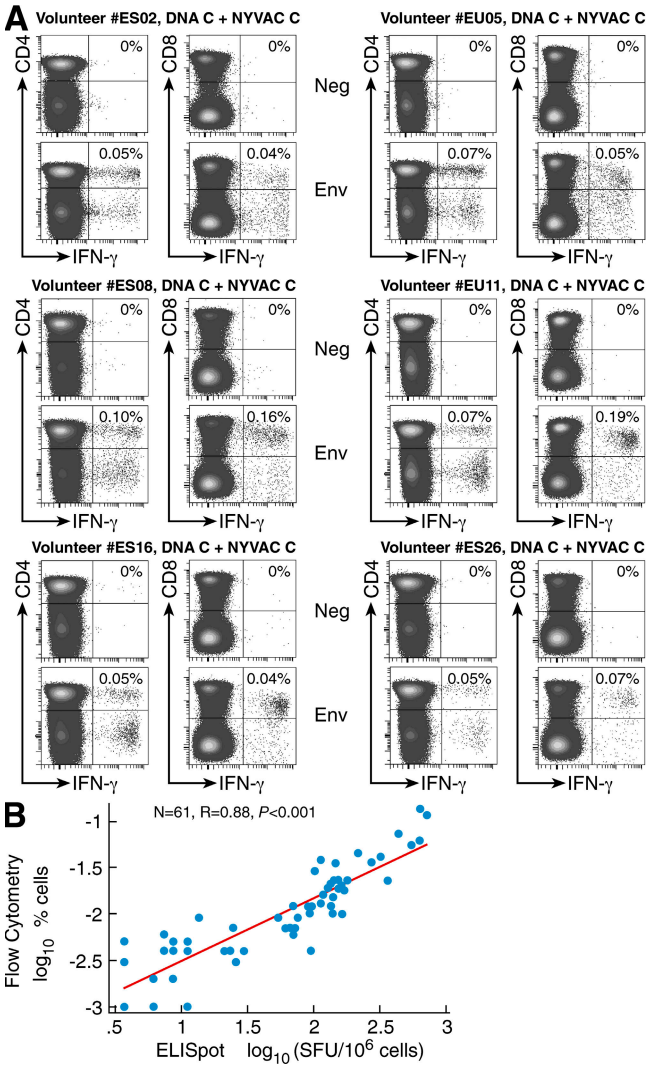

Figure 4. Vaccine-induced CD4 and CD8 T cell responses. (A) Flow cytometry profiles of vaccine-induced CD4 and CD8 T cell responses directed against Env in six out of the seven responders vaccinated with DNA C plus NYVAC C exhibiting CD4 and CD8 T cell responses. CD4 and CD8 T cell responses were defined using polychromatic flow cytometry. Blood mononuclear cells were stimulated with the relevant peptide pools and stained with IFN-γ, CD4, and CD8 antibodies. (B) Correlation of the IFN-γ–secreting cells measured by both flow cytometry and ELISPOT assay. This comparison was performed in the 17 volunteers with vaccine-induced T cell responses in the range of 100 SFU/10^6 cells or above.

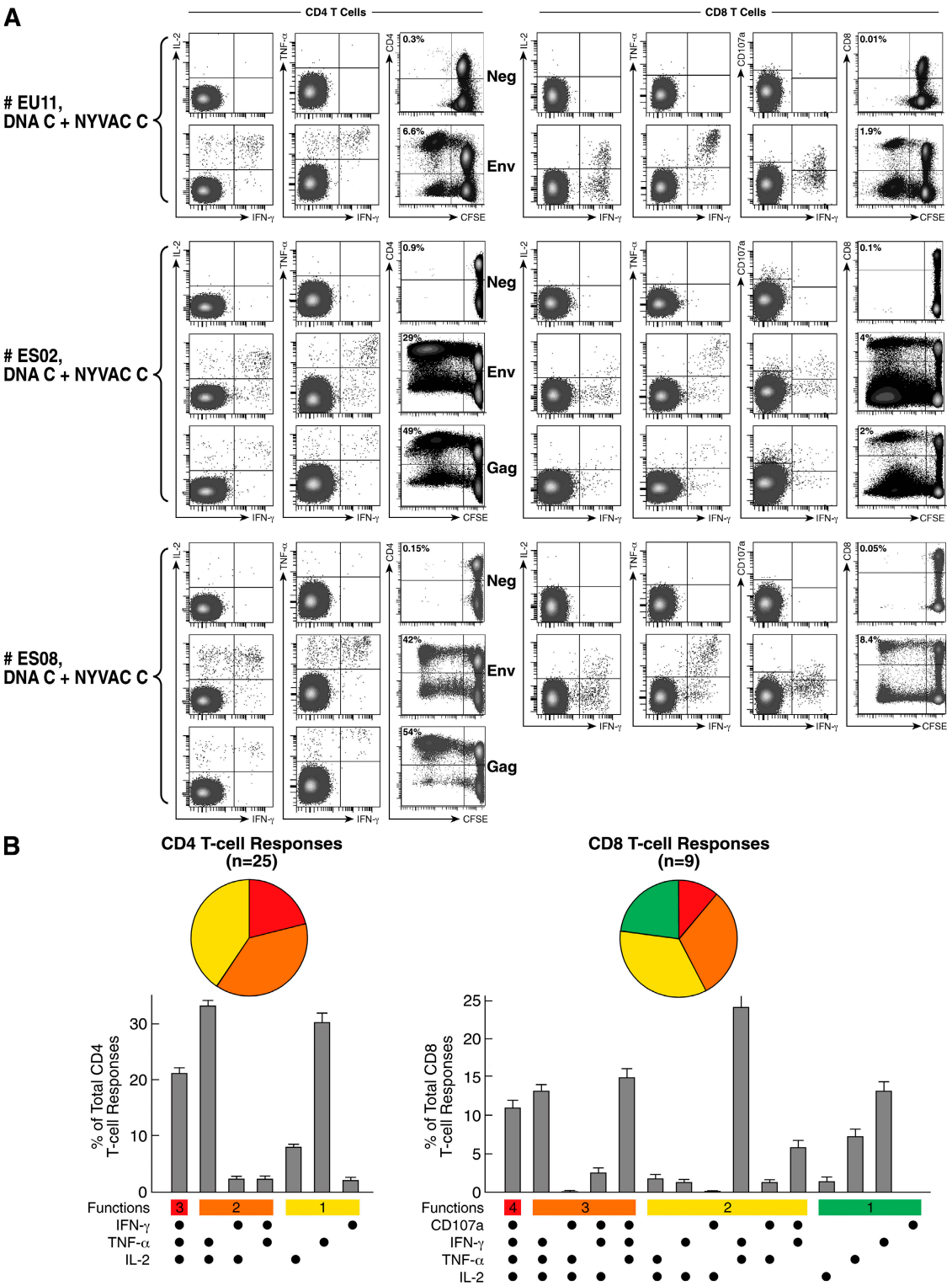

Figure 5. Functional profile of vaccine-induced CD4 and CD8 T cells. (A) Flow cytometry profiles of CD4 T cells and CD8 T cells able to mediate degranulation activity and to secrete IL-2, IFN-γ, and TNF-α are shown. Blood mononuclear cells were stimulated with the relevant peptide pools for 16 h and stained as described in Materials and methods. The functional profiles of both CD4 and CD8 T cells were performed on blood mononuclear cells of volunteers EU11, ES02, and ES08 that were vaccinated with DNA C plus NYVAC C. Volunteers ES02 and ES08 also exhibited Gag-specific in addition to Env-specific CD4 and CD8 T cell responses. With regard to the proliferation, cells were labeled with CFSE and stimulated with the relevant peptide pools, and proliferation was measured at day 6. (B) Functional composition of CD4 and CD8 T cell responses. The results shown were generated from the determinations in 11 responders. All the possible combinations of the responses are shown on the x axis, whereas the percentages of the functionally distinct

Of note, the polychromatic flow cytometry analysis allowed us to provide an independent confirmation of the responses assessed using the IFN-γ ELISPOT assay. The frequencies of IFN-γ–secreting T cells measured by both assays were compared in 17 responders, with a very high correlation between the frequencies measured by the ELISPOT assay and by flow cytometry (Fig. 4 B).

Functional profile of vaccine-induced CD4 and CD8 T cell responses

The panel of T cell functions analyzed included IL-2, TNF-α, and IFN-γ secretion and proliferation for both CD4 and CD8 T cells, and degranulation activity for CD8 T cells. Env- and Gag-specific CD4 and CD8 T cell functions were analyzed using polychromatic flow cytometry (Fig. 5). T cell functions were analyzed after stimulation with Env- or Gag-derived peptide pools. Representative functional profiles are shown in responders EU11, ES02, and ES08 vaccinated with DNA C plus NYVAC C (Fig. 5 A). These three responders had both vaccine-induced CD4 and CD8 T cell responses, and responders ES02 and ES08 also had Gag-specific in addition to Env-specific responses.

The simultaneous analysis of three functions allowed the assessment of the quality of the vaccine-induced CD4 and CD8 T cell responses. On the basis of the analysis of IL-2 and IFN-γ and TNF-α secretion, seven distinct Env- and Gag-specific CD4 T cell populations were identified (Fig. 5 B). Vaccine-induced CD4 T cell responses had a polyfunctional profile, with ~60% of CD4 T cells exhibiting two or three functions (Fig. 5 B). Furthermore, vaccine-induced CD4 T cells efficiently proliferated after stimulation with the Env- and Gag-derived peptide pools (Fig. 5 A).

Similar to CD4 T cells, vaccine-induced CD8 T cells were highly polyfunctional. The simultaneous measure of four functions (IL-2, IFN-γ, and TNF-α secretion, and degranulation activity) allowed the identification of 15 functionally distinct CD8 T cell populations. About 70% of vaccine-induced Env- and Gag-specific CD8 T cells exhibited more than one function (Fig. 5 B). Finally, vaccine-induced CD8 T cells were endowed with proliferation capacity after Env- and Gag-specific stimulation (Fig. 5 A).

Collectively, these results indicated that vaccination with DNA C plus NYVAC C induced polyfunctional Env- and Gag-specific CD4 and CD8 T cell responses.

Phenotypic profile of vaccine-induced CD4 and CD8 T cell responses

Phenotypic analysis of vaccine-induced T cell responses was performed in volunteer ES26 vaccinated with DNA C plus NYVAC C. Both Env-specific CD4 and CD8 T cells were induced after vaccination. Blood mononuclear cells of volunteer ES26 were collected at different time points (weeks 24, 28, and 48), stimulated with Env peptide pools for 16 h, and stained with CD4, CD8, CD45RA, CCR7, IL-2, and IFN-γ antibodies. Previous studies have shown that CD45RA and CCR7 define functionally distinct populations of memory antigen-specific CD4 and CD8 T cells (20–23). The totality (single IL-2 plus dual IL-2/IFN-γ plus single IFN-γ) of Env-specific CD4 T cells was $CD45RA^{-}CCR7^{-}$, and the phenotypic profile and percentage of Env-specific CD4 T cells remained unchanged over time (Fig. 6).

The Env-specific CD8 T cells (dual IL-2/IFN-γ plus single IFN-γ) were almost equally distributed within $CD45RA^{-}CCR7^{-}$ and $CD45RA^{+}CCR7^{-}$ cell populations at week 24 (Fig. 5). However, there was a progressive loss of the $CD45RA^{-}CCR7^{-}$ Env-specific CD8 T cell population over time, and ~90% of the vaccine-induced CD8 T cells were $CD45RA^{+}CCR7^{-}$ at week 48 (Fig. 6).

Of note, the changes in phenotype and in the percentage of Env-specific CD8 T cells were observed only for vaccine-induced CD8 T cells because the phenotype and the percentage of EBV/CMV-specific CD8 T cell responses assessed in blood samples collected at the same time points in volunteer ES26 remained unchanged (Fig. 6). Similar results were obtained in three additional volunteers.

Epitope mapping of vaccine-induced CD4 and CD8 T cell responses

Identification of epitopes recognized by vaccine-induced CD4 and CD8 T cell populations was performed in nine volunteers, eight belonging to the DNA C plus NYVAC C arm and one to the NYVAC C–alone arm. Characterization was limited to the Env-specific responses. After the initial screening using Env-derived peptide pools, identification of the peptides recognized was performed by testing the reactivity of blood mononuclear cells against the relevant peptides in a matrix setting using the IFN-γ ELISPOT assay. After this analysis, 19 different Env-derived peptides were identified in the nine volunteers studied, and further characterization of the vaccine-induced CD4 and CD8 T cell populations recognizing these peptides was performed using polychromatic flow cytometry (Table I and Fig. 7). A variable number of peptides, ranging from 2 to 8, were recognized in each volunteer, with a mean of 4.2 peptides (Fig. 7 A). 10 out of 19 peptides identified in the nine volunteers corresponded to epitopes that have already been described either in subjects with chronic HIV-1 infection or in vaccine studies performed in mice and humans (Table I). However, nine potential novel epitopes were identified that have not been previously described or reported, according to the Los Alamos database (Table I) (24).

cell populations within the total CD4 and CD8 T cell populations are shown on the y axis. Responses are grouped and color-coded on the basis of the number of functions. The pie chart summarizes the data, and each slice of the pie corresponds to the fraction of CD4 or CD8 T cells with a given number of functions within the total CD4 and CD8 T cell populations. Bars correspond to the fraction of different functionally distinct T cell populations within total CD4 and CD8 T cell populations. Mean and standard errors are also shown.

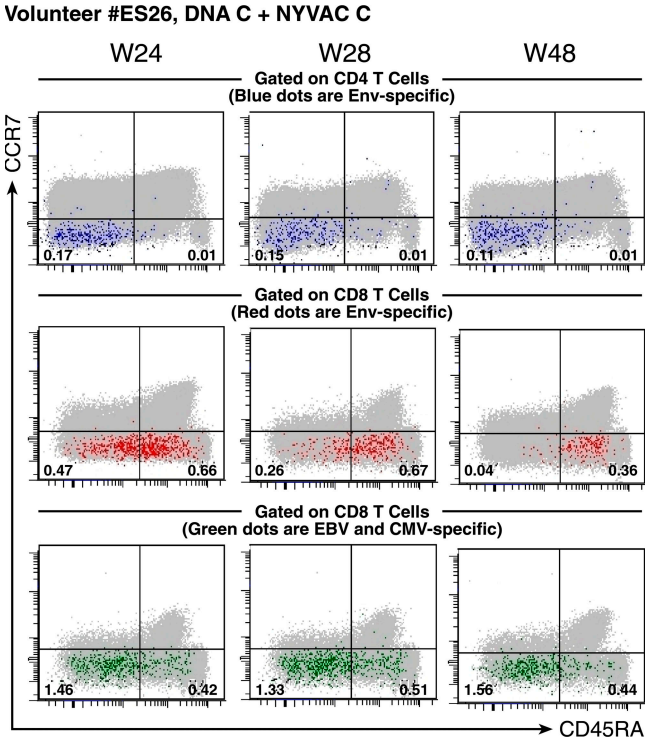

Figure 6. Phenotypic analysis of vaccine-induced CD4 and CD8 T cells. Blood mononuclear cells obtained from volunteer ES26, that was vaccinated with DNA C plus NYVAC C, were stimulated with Env-derived peptide pools or with CMV/EBV-derived peptides for 16 h and stained with IL-2, IFN-γ, CD4, CD8, CD45RA, and CCR7 antibodies. The blue dots indicate Env-specific (IL-2 plus IFN-γ) vaccine-induced CD4 T cells. The red dots indicate Env-specific (IL-2 plus IFN-γ) vaccine-induced CD8 T cells. The green dots indicate CMV/EBV-specific (IL-2 plus IFN-γ) vaccine-induced CD8 T cells.

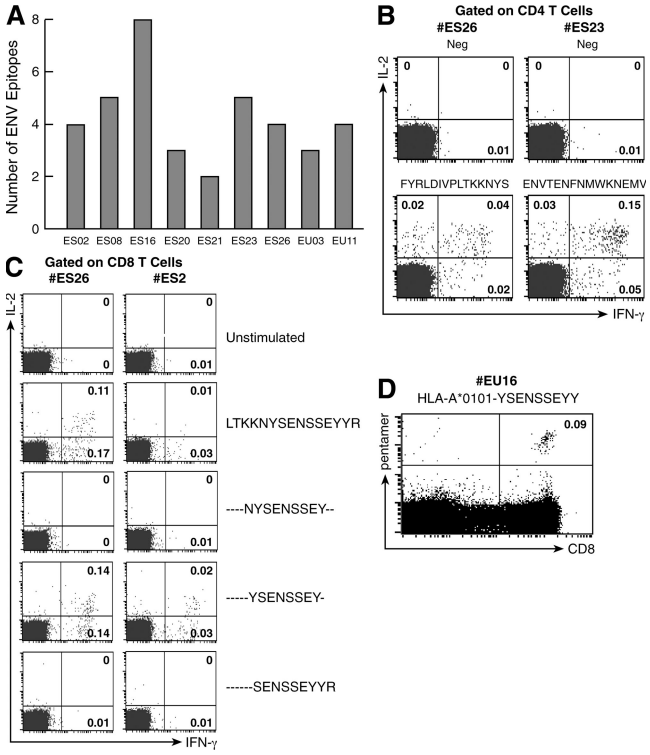

Figure 7. Breadth of vaccine-induced T cell responses against Env. (A) Number of peptides/epitopes recognized in nine volunteers. Env-specific T cell responses were identified using peptide pools in preliminary experiments. The peptides of the relevant pool were then organized in a matrix setting to perform peptide/epitope mapping. Peptide/epitope mapping was performed combining the IFN-γ ELISPOT assay with polychromatic flow cytometry. (B) Example of a peptide/epitope mapping recognized by CD4 T cells. (C) Epitope mapping of the YSENSSEYY CD8 T cell epitope in two representative volunteers vaccinated with DNA C plus NYVAC C. (D) YSENSSEYY-specific CD8 T cells after staining with the relevant HLA-A*0101–YSENSSEYY pentameric complex.

Representative flow cytometry profiles of vaccine-induced Env-specific CD4 and CD8 T cells recognizing individual peptides are shown in Fig. 7 (B and C). In particular, we had the opportunity to perform fine epitope mapping of peptide LTKKNYSENSSEYYR recognized by CD8 T cells in seven volunteers (six belonging to the DNA C plus NYVAC C group and one to the NYVAC C–alone group). By using a set of overlapping peptides, we demonstrated that the epitope recognized by vaccine-induced CD8 T cells corresponded to the following sequence: YSENSSEYY (Fig. 7 C). Two representative examples of the YSENSSEYY epitope mapping in volunteers ES26 and ES2 are shown in Fig. 7 C. The YSENSSEYY epitope was restricted by HLA-A*0101, as demonstrated by the detection of a vaccine-induced well-defined population of YSENSSEYY-specific CD8 T cells using the relevant HLA-A*0101–YSENSSEYY pentameric complex (Fig. 7 D).

Durability of vaccine-induced T cell responses

The duration of the study in the original protocol was 48 wk. However, to gain insights on the long-term durability of the vaccine-induced T cell response, the protocol was subsequently amended to assess the T cell responses at week 72, i.e., 1 yr after the last immunization. The protocol was amended only in Lausanne and, after institutional review board approval, blood was collected at week 72 from volunteers that were originally enrolled at the Lausanne site, which had a positive IFN-γ ELISPOT response at week 48. We analyzed 13 volunteers (11 belonging to the DNA C plus NYVAC C group and 2 to the NYVAC C–alone group) at week 72. None of the two volunteers belonging to the NYVAC C alone group had a positive IFN-γ T cell response at week 72 (Fig. 8). 9 out of the 11 volunteers belonging to the DNA C plus NYVAC C group had a positive IFN-γ T cell response at week 72. Of interest, the magnitude of the IFN-γ T cell response observed at week 72 was not significantly different ($P = 0.09$) from that measured in the eight volunteers with responses at all three time points (Fig. 8, A and B).

The changes in the composition of vaccine-induced, functionally distinct CD4 and CD8 T cell responses were compared at weeks 28, 48, and 72 by performing the simultaneous measures of multiple functions using polychromatic flow cytometry.

Table I. List of env epitopes

HLA restriction	Sequence	Region	Previous description[a]
Class II	VGNLWVTVYYGVPVW	C1/C2	VYYGVPVWKEA
	WVTVYYGVPVWKGAT		
	GATTTLFCASDAKAY	C1/C2	Not described
	TTLFCASDAKAYDTE		
	THACVPADPNPQEMV	C1/C2	CVPTDPNPQEVV
	ENVTENFNMWKNEMV	C1/C2	PQEVVLVNVTENFNMWKNDMV
	ENFNMWKNEMVNQMQ		
	EMVNQMQEDVISLWD	C1/C2	Not described
	CVKLTPLCVTLECRN	C1/C2	Not described
	NCSFNATTVVRDRKQ	V1/V2	Not described
	NATTVVRDRKQTVYA		
	VYALFYRLDIVPLTK	V1/V2	Not described
	FYRLDIVPLTKKNYS		
	INCNTSAITQACPKV	C3	KLTSCNTSVITQACPKVSFE
	FDPIPIHYCTPAGYA	C3	PKVSFEPIPIHYCAPAGFAILKCNN
	PKVTFDPIPIHYCTP	C3	PKVSFEPIPIHYCAPAGFAILKCNN
	FDPIPIHYCTPAGYA		
	TGDIIGDIRQAHCNI	V3/C4	GRAFVTIGKIGNMRQAHCNISRAKWNAT
	SSSIITIPCRIKQII	V4/C5	Not described
	ITIPCRIKQIINMWQ	C5	DTITLPCRIKQIINMWQKVG
	CRIKQIINMWQEVGR		
	VGRAMYAPPIKGNIT	C5	VGKAMYAPPISGQIRCSSNITGLL
	MYAPPIKGNITCKSN		
	PIKGNITCKSNITGL		
	ETFRPGGGDMRNNWR	C5	FRPGGGDMRDNWRSEL
	ELYKYKVVEIKPLGV	C5	Not described
	YKVVEIKPLGVAPTT		
Class I	EIKPLGVAPTTTKRR	C5	Not described
	LGVAPTTTKRRVVER		
HLA-A*01	YSENSSEYY	V1/V2	Not described

[a]According to Los Alamos Database (reference 24).

No substantial changes were observed in the frequency of the seven functionally distinct CD4 T cell populations over time (Fig. 8 C). Only a drop in the range of 30% was observed in the polyfunctional (IL-2/IFN-γ/TNF-α and IL-2/TNF-α) CD4 T cell populations between weeks 28 and 48, whereas the frequency of these populations remained stable between weeks 48 and 72 (Fig. 8 C).

With regard to the vaccine-induced CD8 T cell responses, a decrease in the frequency of functionally distinct CD8 T cell populations was observed over time (Fig. 8 C). In particular, three CD8 T cell populations (dual IFN-γ/TNF-α, dual CD107a/ IFN-γ, and single IFN-γ) decreased >50% between weeks 28 and 72 (Fig. 8 C).

Vaccine-induced antibody response against gp-140

Vaccine-induced IgG antibodies against gp140 CN54 were assessed at different time points during the immunization regimen. The induction of IgG anti-gp140 CN54 was assessed in an ELISA assay. Only a small number of volunteers (25%) had a measurable antibody response at week 26 in the NYVAC C–alone group, whereas a large percentage (75%) of volunteers had measurable IgG anti-gp140 antibodies in the DNA C plus NYVAC C group ($P = 0.007$; not depicted). In addition to the differences in the percentage of responders between the two study groups, the magnitude of the antibody response was also significantly greater in the DNA C plus NYVAC C group compared with the NYVAC C–alone group ($P = 0.006$; not depicted). Vaccine-induced antibodies failed to show any neutralizing activity in the three neutralizing assays tested (see Materials and methods for the description of the assays; not depicted).

DISCUSSION

Previous prime-boost clinical studies of candidate T cell vaccines for HIV have suggested that adenovirus vectors were more immunogenic than poxvirus vectors in terms of cellular immune responsiveness. In the EV02 trial, we demonstrate that DNA plus NYVAC is a highly immunogenic prime-boost regimen, with durable responses up to 1 yr after vaccination. The primary analysis of the vaccine-induced T cell responses was

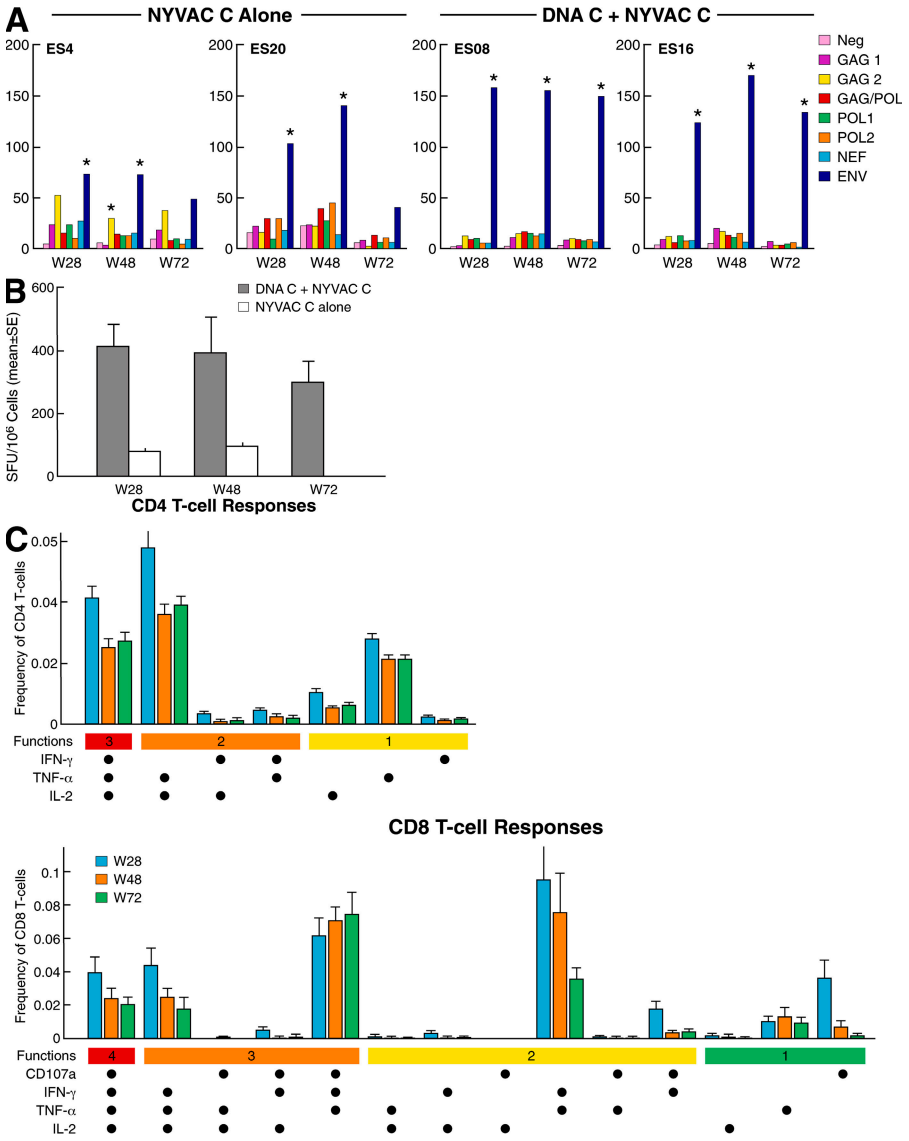

Figure 8. Durability of vaccine-induced T cell responses. (A) Monitoring of the T cell responses in representative volunteers of the two study groups. Only volunteers with positive T cell responses in the IFN-γ ELISPOT assay at week 48 were retested at week 72, which corresponded to 1 yr after the completion of the vaccination regimen. (B) Mean frequencies of IFN-γ–secreting cells/10^6 blood mononuclear cells at weeks 28, 48, and 72 in the eight volunteers within the DNA C plus NYVAC C group with a positive T cell response at weeks 72, 48, and 28, and mean frequencies of IFN-γ–secreting cells/10^6 blood mononuclear cells in the two volunteers within the NYVAC C group alone with responses at weeks 48 and 28. (C) Changes in the frequencies of functionally distinct CD4 and CD8 T cell populations over time. The results shown were generated from the determinations in six responders. The functional profile of vaccine-induced CD4 and CD8 T cells was assessed using polychromatic flow cytometry as described in Materials and methods. The functional composition of CD4 and CD8 T cell responses was determined as described in Fig. 5 B. All the possible combinations of the responses are shown on the x axis, whereas the percentage of the functionally distinct CD4 and CD8 T cell populations at weeks 28, 48, and 72 are shown on the y axis.

performed using a validated IFN-γ ELISPOT assay. Polychromatic flow cytometry was used to characterize the phenotypic and the functional profiles of the vaccine-induced T cell populations. Furthermore, both the IFN-γ ELISPOT assay and polychromatic flow cytometry were instrumental in analyzing the breadth of the vaccine-induced T cell responses and for performing epitope mapping. All the analyses have been performed on frozen blood mononuclear cell samples, and the evaluation of the immunogenicity has been assessed in the IFN-γ ELISPOT assay after a conventional overnight stimulation of the blood mononuclear cells, with the panel of peptide pools encompassing Env, Gag, Pol, and Nef of HIV-1 clade C CN54.

The majority (18 out of 20, 90%) of the volunteers immunized with the DNA C plus NYVAC C regimen had a positive response using the IFN-γ ELISPOT assay at weeks 26 or 28, the primary endpoints of the study. The percentage of responders in the NYVAC C–alone group was 33%. These results were

superior to previous studies that have evaluated the immunogenicity of DNA plus poxvirus (MVA) vaccine regimens in which the percentage of responders was 17% (low dose DNA) and ~40% (high dose DNA) when conventional overnight stimulation was performed to analyze IFN-γ–secreting cells (as used in EV02) (16, 17). Responses were observed in the eight volunteers in the high dose DNA plus MVA study only using a cultured ELISPOT assay (5-d stimulation) able to detect weak vaccine-induced T cell responses (16, 17). The immunogenicity observed in EV02 was also higher than that observed in studies using Ad5-based vaccine strategies and in the same range of studies using DNA plus Ad5 regimens (unpublished data).

Vaccine-induced IFN-γ–secreting cells were detected only in 7 out of 20 volunteers after DNA C vaccination and before NYVAC C boosting. However, despite the fact that vaccine-induced T cell responses were not measurable in the majority of volunteers after the two DNA vaccinations, the DNA priming was clearly responsible for the substantial increase in the immunogenicity and magnitude (see below) of the vaccine-induced T cell responses in the DNA C plus NYVAC C group compared with the NYVAC C–alone group. These results indicate that the assessment of immunogenicity after immunization with DNA alone is not a reliable measure of the priming ability of DNA candidate vaccines.

Collectively, these results indicate that the DNA C plus NYVAC C regimen is superior to the NYVAC C–alone regimen and has a degree of immunogenicity comparable to that of other promising candidate vaccines that have entered large phase II/IIB clinical studies (3, 4, 13).

The magnitude of the vaccine-induced T cell responses as measured by the frequency of IFN-γ–secreting cells using the ELISPOT assay was substantially higher (three- to fourfold) in the DNA C plus NYVAC C group compared with the NYVAC C–alone group. The magnitude of the vaccine-induced T cell responses observed after DNA C plus NYVAC C immunization was superior to that observed in previous DNA plus MVA studies (17) and comparable to that of Ad5 candidate vaccines and to the DNA plus Ad5 vaccine combination (unpublished data).

The vaccine-induced T cell responses were predominantly (in 100% of responders) mediated by CD4 T cells. However, CD8 T cell responses were also found in 47% of responders. Furthermore, both vaccine-induced CD4 and CD8 T cell responses were predominantly directed against Env (the median magnitude of Env T cell responses in the DNA C plus NYVAC C group vs. the NYVAC C–alone group was 299 and 131 SFU/10^6 cells, respectively). T cell responses against Gag, Pol, and Nef were detected in 48% of volunteers and had lower magnitude (the median magnitude of Gag-, Pol-, and Nef-specific T cell responses was ~100 SFU/10^6 cells).

The finding of predominant CD4 T cell responses after immunization with DNA C plus NYVAC C is consistent with previous studies (3, 4, 17) investigating the T cell responses induced by poxvirus candidate vaccines. The predominant CD4 T cell response after immunization with vaccines including poxviruses seems to differentiate these latter from Ad5-based candidate vaccines and the DNA plus Ad5 vaccine regimen that appear to induce more balanced CD4 and CD8 T cell responses (unpublished data). Three factors may have contributed to the dominant CD4 T cell and Env-specific responses: (a) the DNA priming that seems to favor the development of the CD4 T cell response; (b) the monovalent nature of the NYVAC C construct in which Env, Gag, Pol, and Nef are expressed within the same vector; and (c) the construction of the NYVAC vector with regard to Env, which is expressed in a secreted form. The use of multivalent instead of monovalent DNA and Ad5-based vaccines may reduce the Env immunodominance in favor of more balanced Env, Gag, and Pol responses. The secreted form of Env is likely to be responsible for favoring the exogenous pathway of antigen presentation and thus stimulation of CD4 T cells.

In natural infection studies, it has been reported that Gag-specific CD8 T cell responses are associated with better control of HIV disease in individuals with chronic HIV-1 infection, whereas Env-specific CD8 T cell responses are associated with poor control (25). However, it is not clear from this study whether (a) Gag-specific CD8 T cell responses are the cause or rather the effect of lower levels of virus replication, and (b) results generated in the nonhuman primate model have shown that the presence of Env- and Gag-specific vaccine-induced T cell responses in animals immunized with DNA plus Ad5 expressing Env and Gag conferred better protection from disease after infection compared with animals immunized with DNA plus Ad5 Gag-expressing vaccines (26).

The DNA C plus NYVAC C vaccine regimen induced CD4 T cell responses in 100% of the immunized individuals and CD8 T cell responses in 50% of vaccines. In this regard, it is important to mention that the presence of the vaccine-induced CD4 T cell responses strongly correlated with the containment of viremia in macaques exposed to the highly pathogenic SIV_{mac251} after immunization with the DNA-SIV-*gag-env* plus NYVAC-SIV-*gag-pol-env* (27). Therefore, vaccine-induced CD4 T cell responses may be effective in the attenuation of HIV disease.

Env- and Gag-specific CD4 and CD8 T cell responses induced by DNA C plus NYVAC C vaccination were polyfunctional. Recently (8, 10, 11), the term polyfunctional has been used to define T cell responses that, in addition to typical effector functions such as secretion of IFN-γ, TNF-α, and MIP-1β, as well as cytotoxic activity, comprise T cell populations also able to secrete IL-2 and retain antigen-specific proliferation capacity, whereas the term "only-effector" defines T cell responses/populations with typical effector functions but lacking IL-2 and proliferation capacity. Of interest, several studies (11, 28–31) have demonstrated that polyfunctional and not only-effector T cell responses were associated with protective antiviral immunity (32). The DNA C plus NYVAC C immunization therefore induced the best functional profile of virus-specific CD4 and CD8 T cells capable of controlling virus replication in several chronic virus infections, such as cytomegalovirus, Epstein-Barr virus, herpes simplex virus, and

HIV-1 in patients with nonprogressive disease (8, 10, 33). These data are consistent with our previous study demonstrating that vaccine-induced CD8 T cells after vaccination with DNA C plus NYVAC C were highly polyfunctional and that almost 75% of these cells had four or five functions based on IFN-γ, TNF-α, MIP-1β, IL-2, and CD107a (34).

The polyfunctional vaccine-induced CD4 T cell populations were $CD45RA^{-}CCR7^{-}$, a phenotype of cells with effector functions but also with the ability to secrete IL-2 and endowed with proliferation capacity (20–23, 29, 30, 33). The phenotype of the vaccine-induced CD4 T cells remained unchanged over time. The vaccine-induced CD8 T cells were either $CD45RA^{-}CCR7^{-}$ or $CD45RA^{+}CCR7^{-}$. This phenotypic profile defines memory CD8 T cells at intermediate and advanced stages of differentiation and is consistent with our recent study using CD27 and CD45RO (34). The presence and rapid appearance after immunization of $CD45RA^{+}CCR7^{-}$ CD8 T cells is of interest. This phenotype has been proposed to define effector CD8 T cells at advanced stages of differentiation (21). Furthermore, virus-specific $CD45RA^{+}CCR7^{-}$ CD8 T cells have been found in controlled chronic virus infections such as CMV and EBV (21, 29, 30), and a correlation between the percentage of this cell population and virus control has also been shown in HIV-1 infection (8). The majority of the vaccine-induced $CD45RA^{-}CCR7^{-}$ CD8 T cell population disappeared over time, whereas ~90% of vaccine-induced CD8 T cells were $CD45RA^{+}CCR7^{-}$ at week 48, 6 mo after the last immunization. Therefore, the DNA C plus NYVAC C vaccination likely induces the generation of a long-lived population of memory CD8 T cells.

Of interest, the DNA C plus NYVAC C vaccination induced a broad T cell response, with a mean 4.2 epitopes recognized per volunteer. About 50% of epitopes identified in the EV02 study have not previously been described according to the Los Alamos database (24), and approximately two out of three of the total epitopes identified were located in constant regions.

A critical component of the effectiveness of vaccines is their ability to induce long-lasting immunity. The results obtained in EV02 study after vaccination with DNA C plus NYVAC C are extremely promising. More than 70% of volunteers have still measurable vaccine-induced T cell responses 1 yr after the last vaccination and, more importantly, the magnitude of the T cell responses is not substantially changed compared with 1 mo after the completion of the vaccination regimen.

Poxvirus vectors have traditionally represented an interesting platform in the HIV vaccine arena because of (a) their large and successful use in the veterinary field, (b) extensive safety data in humans, (c) their use in vaccine platforms for other infectious diseases and cancer, and (d) their facility to be manipulated and to be inserted with large gene fragments (4). However, disappointing results on their immunogenicity in humans had seriously cast doubt on the validity of the poxvirus platform and the rationale for their further development in the HIV vaccine arena. The present data generated within the EV02 study have clearly demonstrated that a poxvirus candidate vaccine, NYVAC, in combination with DNA is highly immunogenic, induces vigorous and broad T cell responses, comprising of both CD4 and CD8 T cell responses, which are polyfunctional, and more importantly, this vaccine regimen induces long-lasting T cell immunity.

These promising results support the further development of the poxvirus platform and the move of the DNA C plus NYVAC C vaccine regimen into larger clinical trials.

MATERIALS AND METHODS

DNA immunogens GagPolNef (GPN) and Env. RNA- and codon-optimized GPN and Env gene vector inserts were designed by GENEART AG using the GeneOptimizer software package. Clade B/C' GPN and Env (gp120) sequences were designed based on sequence information derived from a 97CN54 provirus clone (sequence submitted to GeneBank) (35). The HIV C clade isolate CRF_70 B/C' 97CN54 was collected from Sinkiang Province in China, biologically characterized, and sequenced in a collaboration of China CDC (the Chinese Academy for Preventive Medicine) and the European Commission–funded research cluster CHIVAC 1–3 in an attempt to generate region-specific candidate vaccines. This and subsequently detected, closely related variants represent at least 60% of HIV infections in China and are anti-genetically also close to other C clade isolates from India and South and East Africa. The CN54 Env construct comprises 1,500 nucleotides encoding an artificial signal peptide (MDRAKLLLLL LLLLPQAQ), followed by gp120 CN54 (nucleotides 5,673–7,109). The 5′ part of the 4,254 nucleotide CN54 GPN polygene construct encodes the group-specific antigen (nucleotides 167–1,651) with a G2A modification rendering this polyprotein myristylation deficient. The Gag coding sequence is followed by a 952-bp (nucleotides 1,444–2,406) fragment encoding the 5′ part of *pol*, including a D577N mutation leading to an inactivation of the viral protease. A 618-bp fragment encoding a scrambled Nef variant (5′ end of nucleotides 8,170–8,469 linked to 3′ end of nucleotides 8,470–8,787) was fused to the 3′ end of *pol*-coding sequence replacing the active site of the reverse transcription. The 3′ pol reading frame (nucleotides 2,527–3,591) lacking the integrase gene was extended by the 3′ end of the scrambled Nef gene. The sequence stretch (nucleotides 2,407–2,514) encoding the active site of the reverse transcription (amino acids 1,382–1,417 in GPN) was translocated to the 3′ end of the polygene construct, resulting in an open reading frame encoding the ~160-kD nonglycosylated artificial GPN polyprotein. Both genes were placed under direct control of CMV IE promoter/enhancer to generate pORT1a-GPN and pORT1a-gp120, lacking any antibiotic resistance gene and instead using a repressor titration system for plasmid selection (Cobra Biomanufacturing Plc.) (36). The genetic stability of both plasmids was evaluated in a DH1lacdapD host strain up to 39 cell generations and controlled by double-strand DNA sequencing (GENEART AG).

Construction of recombinant NYVAC vector. The NYVAC vector expressing *Gag/Pol/Nef* and *Env* of clade C HIV-1 97CN54 was used as described previously (35). Plasmids containing codon-optimized clade C HIV-1 *gagpolnef* and *gp120* genes (pMA60gp120C/gagpolnef-C-14) were used. Functional expression of the donor genes has been demonstrated by Western blot analysis of the proteins produced by the premaster seed lot. Expression levels of the *GagPolNef* polygene and the *Env* gene were similar. The description of the recombinant NYVAC vector expressing Env, Gag, Pol, and Nef has been described elsewhere (37). The characteristics and immunogenic potential of NYVAC C has been documented in mice (37).

DNA and NYVAC HIV-1 clade clinical lots. Good manufacturing production clinical lots of DNA C and NYVAC C were manufactured by Cobra Biomanufacturing Plc. and sanofi pasteur.

Immunization schedule. At weeks 0 and 4, one group was "primed" with DNA, and one group received nothing. At weeks 20 and 24, all volunteers were immunized with NYVAC. The DNA pORT-gp120 and pORT-gpn

plasmids were mixed before administration in an equimolar fashion (final total DNA concentration, 1.05 mg/ml). Volunteers were injected i.m. with 2 ml DNA in each upper leg (4.2 mg/4 ml total per person). The NYVAC vector expressing GPN and Env was administered i.m. in the left upper arm ($10^{7.7}$ pfu/ml total). The study was approved by the institutional review boards of the Centre Hospitalier Universitaire Vaudois, University of Lausanne, Switzerland, and of St Mary's Hospital, Imperial College, London.

Synthetic peptides and peptide–MHC class I complex. All peptides used in this study were HPLC purified (>80% purity). Overlapping peptides (15 mers with 11 amino acids overlap; $n = 474$) spanning the entire *Gag/Pol/Nef* polygene, and the Env clade C of HIV-1 97CN54 (Synpep Corporation) was grouped in eight pools as follows: Gag1 60 peptides (Cg1-Cg240), Gag2 61 peptides (Cg244-Cg486), Gag/Pol 60 peptides (Cgp485-Cp721), Pol1 61 peptides (Cp725-Cpn817 and Cnp1017-Cp1161), Pol2 61 peptides (Cp1165-Cp1403), Nef 49 peptides (Cn838-Cnp1030), Env1 49 peptides (CN9-CN249), and Env2 63 peptides (CN253-CN485). In addition, Env peptides were either rearranged in a matrix setting for the fine epitope mapping analyses or used as single peptides. NYSENSSEY, YSENSSEYY, and SENSSEYYR were obtained from the peptide facility at the University of Lausanne. Furthermore, a set of peptides ($n = 28$) most frequently recognized in CMV, EBV, and flu infection (CEF pool) (38, 39) was used as an additional positive control. The HLA-A*0101-YSENSSEYY peptide–MHC pentameric complex was purchased from PROIMMUNE.

ELISPOT assays. ELISPOT assays were performed at weeks 0, 5, 20, 24, 26, 28, and 48. In addition, 13 volunteers recruited in Lausanne were also evaluated at week 72. ELISPOT assays were performed as per the manufacturer's instructions (BD Biosciences). In brief, cryo-preserved blood mononuclear cells were rested for 8 h at 37°C, and then 200,000 cells were stimulated with peptide pools (1 μg of each single peptide) in 100 μl of complete media (RPMI plus 10% FBS) in quadruplicate conditions. Media only was used as negative control. 200 ng/ml of staphylococcal enterotoxin B (SEB) was used as a positive control on 50,000 cells, and stimulation with the CEF pool (on 200,000 cells) was used as an additional (antigen-specific) positive control. Results are expressed as the mean number of SFU/10^6 cells from quadruplicate assays. Only cell samples with >80% viability after thawing were analyzed, and only assays with <50 SFU/10^6 cells for the negative control and >500 SFU/10^6 cells after SEB stimulation were considered valid.

Flow cytometry analysis. Cryo-preserved blood mononuclear cells ($1–2 \times 10^6$) were stimulated overnight in 1 ml of complete media containing 1μl/ml Golgiplug (BD Biosciences) and 0.5 μg/ml αCD28 antibodies (BD Biosciences) as described previously (29). For stimulation of blood mononuclear cells, individual peptides or peptide pools were used at 1 μg/ml for each peptide. SEB stimulation (200 ng/ml) served as positive control. For functional analyses (i.e., intracellular cytokine staining [ICS] and assessment of the degranulation activity/CD107a mobilization), the following antibodies were used in various combinations: CD4-FITC, -PerCP-Cy5.5, or -PB; CD8-PerCP-Cy5.5, -PB, or APC-Alexa 700; CD3-ECD; CD14-PB; CD16-PB; CD19-PB; IFN-γ-FITC or -APC; IL-2-PE or -APC; TNF-α-FITC or -PECY-7; and CD107a-FITC or -PE. All antibodies were from BD Biosciences, except CD8-APC-Alexa 700 (VWR International AG) and CD3-ECD (Beckman Coulter). Furthermore, dead cells were excluded using the violet LIVE/DEAD stain kit (Invitrogen). In addition, for phenotypic analyses, the following antibodies were used: CCR7-FITC, -APC, or -APC-Alexa-647; CD45RA-ECD or -PerCP; and CD127-PE or -APC-Alexa-647. CD45RA-ECD and CD127-PE were from Beckman Coulter. At the end of the stimulation period, cells were washed, permeabilized (FACS Perm 2 solution; BD Biosciences) and stained as described previously (29). Data were acquired on a FACSCalibur or on an LSRII three-laser (488, 633, and 405 nm) and analyzed using CELLQuest and DiVa software (Becton Dickinson), respectively. Of note, analyses were also performed using SPICE 4.1.5 software from Mario Roederer, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health. The number of lymphocyte-gated events ranged between 10^5 and 10^6 in the flow cytometry experiments shown. With regard to the criteria of positivity of ICS, the background in the unstimulated controls never exceeded 0.01 to 0.02%. An ICS to be considered positive had to have a background of <20% of the total percentage of cytokine$^+$ cells in the stimulated samples.

Ex vivo proliferation assay. After an overnight rest, cryo-preserved blood mononuclear cells were washed twice, resuspended at 10^6/ml in PBS, and incubated for 7 min at 37°C with 0.25 μM CFSE (Invitrogen) as described previously (29). The reaction was quenched with 1 vol of FCS, and cells were washed and cultured in the presence of 0.5 μg/ml αCD28 antibody (BD Biosciences). $1–2 \times 10^6$ cells were then stimulated with HIV-1 peptide or peptide pools (20 ng/ml of each peptide). SEB stimulation (40 ng/ml) served as a positive control. At day 5, cells were harvested and stained with CD4-PerCP-Cy5.5 and CD8-APC. Cells were fixed with CellFix (BD Biosciences) and acquired on an LSRII. The number of lymphocyte-gated events ranged between 10^5 and 10^6.

Analysis of antibody responses. The induction of HIV-specific antibodies was assessed using an ELISA assay. In brief, 1 μg/ml of recombinant gp140 of CN54 (provided by S. Jeffs, Imperial College, London, UK) in 100 mM Na_2HCO3, pH 9.6, was coated on plates (Maxisorp; Nunc) for one night at 4°C. After elimination of the solution and blocking step with PBS, 5% nonfat dry milk, serum dilutions made in PBS, 5% milk, and 3% Tween 20 (beginning at 1/20) were added for 1 h at 37°C. Antibodies bound to the coated gp140 were revealed by an anti–human IgG–horseradish peroxidase conjugate (1/25 000, A1070; Sigma-Aldrich). The inhibitory activity of antibodies was assessed with three different assays. First, a multiple-round neutralization assay on PBMCs was performed with the homologous primary isolate CN54 using experimental conditions described previously (40). Second, antibody effects in a single-cycle infection of primary isolate Bx08 in the engineered cell line (TZMbl) was measured as reported (41). Third, the inhibition by antibodies of HIV-1 Bal multiplication in macrophages was determined according to the method developed previously (42).

Statistical analysis. An ELISPOT result was defined as positive if the number of SFU was ≥55 SFU/10^6 cells and greater or equal to fourfold the negative control. The primary immunogenicity endpoint was a positive ELISPOT result at weeks 26 and 28. Each participant was classified as a responder if there was at least one positive response against any of the HIV peptides at weeks 26 or 28, and as a nonresponder if responses at these weeks were all negative. The magnitude of a ELISPOT response was described as the sum of SFU of all positive responses and—assuming that there is no overlap in response across the eight peptide pools—expressed per 10^6 cells, either by peptide pool or overall, without subtraction of background. An ICS was considered as positive if background was <20% of the total percentage of cytokine$^+$ cells in the stimulated samples. Of note, the background in the unstimulated controls never exceeded 0.01 to 0.02%. An antibody response was classified as present if the OD measured with sera collected after immunization was at least three times greater than the OD obtained with the corresponding preimmune serum.

Comparisons of categorical variables (e.g., the primary endpoint) were made using Fisher's exact test. The magnitude of an ELISPOT response and other continuous variables was compared between groups using nonparametric statistical tests (magnitude over time, paired Wilcoxon or Friedman test; comparison between randomization groups, Mann-Whitney test). For the comparison of flow cytometry and ELISPOT assay in measuring the frequency of IFN-γ–secreting T cells, generalized estimating equations modeling was used to consider within-participant dependencies. The level of statistical significance was 5% for all analyses, without adjustment for multiple comparisons.

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REFERENCES

1. Schwartländer, B., J. Stover, N. Walker, L. Bollinger, J.P. Gutierrez, W. McGreevey, M. Opuni, S. Forsythe, L. Kumaranayake, C. Watts, and S. Bertozzi. 2001. AIDS. Resource needs for HIV/AIDS. *Science*. 292:2434–2436.
2. Bart, P.A., A. Harari, and G. Pantaleo. 2006. Clinical studies of experimental vaccines. *Current Opinion in HIV & AIDS*. 1:286–293.
3. Letvin, N.L. 2006. Progress and obstacles in the development of an AIDS vaccine. *Nat. Rev. Immunol.* 6:930–939.
4. McMichael, A.J. 2006. HIV vaccines. *Annu. Rev. Immunol.* 24:227–255.
5. Heeney, J.L. 2004. Requirement of diverse T-helper responses elicited by HIV vaccines: induction of highly targeted humoral and CTL responses. *Expert Rev. Vaccines*. 3:S53–S64.
6. Desrosiers, R.C. 2004. Prospects for an AIDS vaccine. *Nat. Med.* 10: 221–223.
7. Pitisuttithum, P., P.W. Berman, B. Phonrat, P. Suntharasamai, S. Raktham, L.O. Srisuwanvilai, K. Hirunras, D. Kitayaporn, J. Kaewkangwal, S. Migasena, et al. 2004. Phase I/II study of a candidate vaccine designed against the B and E subtypes of HIV-1. *J. Acquir. Immune Defic. Syndr.* 37:1160–1165.
8. Pantaleo, G., and R.A. Koup. 2004. Correlates of immune protection in HIV-1 infection: what we know, what we don't know, what we should know. *Nat. Med.* 10:806–810.
9. Schmitz, J.E., M.J. Kuroda, S. Santra, V.G. Sasseville, M.A. Simon, M.A. Lifton, P. Racz, K. Tenner-Racz, M. Dalesandro, B.J. Scallon, et al. 1999. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science*. 283:857–860.
10. Pantaleo, G., and A. Harari. 2006. Functional signatures in antiviral T-cell immunity for monitoring virus-associated diseases. *Nat. Rev. Immunol.* 6:417–423.
11. Betts, M.R., M.C. Nason, S.M. West, S.C. De Rosa, S.A. Migueles, J. Abraham, M.M. Lederman, J.M. Benito, P.A. Goepfert, M. Connors, et al. 2006. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood*. 107:4781–4789.
12. Koopman, G., D. Mortier, S. Hofman, H. Niphuis, Z. Fagrouch, S. Norley, G. Sutter, P. Liljestrom, and J.L. Heeney. 2004. Vaccine protection from CD4+ T-cell loss caused by simian immunodeficiency virus (SIV) mac251 is afforded by sequential immunization with three unrelated vaccine vectors encoding multiple SIV antigens. *J. Gen. Virol.* 85:2915–2924.
13. Johnston, M.I., and A.S. Fauci. 2007. An HIV vaccine–evolving concepts. *N. Engl. J. Med.* 356:2073–2081.
14. Shiver, J.W., and E.A. Emini. 2004. Recent advances in the development of HIV-1 vaccines using replication-incompetent adenovirus vectors. *Annu. Rev. Med.* 55:355–372.
15. Singh Sandhu, D., and J. Tartaglia. Poxvirus as immunization vehicles. *In* Vaccines fourth edition. S.A. Plotkins and W.A. Orenstein, editors. 2004. Sauders, Philadelphia. 1297–1316.
16. Cebere, I., L. Dorrell, H. McShane, A. Simmons, S. McCormack, C. Schmidt, C. Smith, M. Brooks, J.E. Roberts, S.C. Darwin, et al. 2006. Phase I clinical trial safety of DNA- and modified virus Ankara-vectored human immunodeficiency virus type 1 (HIV-1) vaccines administered alone and in a prime-boost regime to healthy HIV-1-uninfected volunteers. *Vaccine*. 24:417–425.
17. Peters, B.S., W. Jaoko, E. Vardas, G. Panayotakopoulos, P. Fast, C. Schmidt, J. Gilmour, M. Bogoshi, G. Omosa-Manyonyi, L. Dally, et al. 2007. Studies of a prophylactic HIV-1 vaccine candidate based on modified vaccinia virus Ankara (MVA) with and without DNA priming: effects of dosage and route on safety and immunogenicity. *Vaccine*. 25:2120–2127.
18. Franchini, G., S. Gurunathan, L. Baglyos, S. Plotkin, and J. Tartaglia. 2004. Poxvirus-based vaccine candidates for HIV: two decades of experience with special emphasis on canarypox vectors. *Expert Rev. Vaccines*. 3:S75–S88.
19. Bart, P.A., A. Harari, C. Cellerai, D. Ciuffreda, T. Barber, W. Stoëhr, S. McCormack, G. Pantaleo, J. Weber, and E. Prgm. 2007. EV02, A phase I trial to assess the safety and immunogenicity of DNA C followed by NYVAC C (IM) in an open, randomised comparison to NYVAC C alone in healthy volunteers at low risk of HIV infection. CROI Meeting Paper 101.
20. Sallusto, F., J. Geginat, and A. Lanzavecchia. 2004. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu. Rev. Immunol.* 22:745–763.
21. Champagne, P., G.S. Ogg, A.S. King, C. Knabenhans, K. Ellefsen, M. Nobile, V. Appay, G.P. Rizzardi, S. Fleury, M. Lipp, et al. 2001. Skewed maturation of memory HIV-specific CD8 T lymphocytes. *Nature*. 410:106–111.
22. Harari, A., F. Vallelian, and G. Pantaleo. 2004. Phenotypic heterogeneity of antigen-specific CD4 T cells under different conditions of antigen persistence and antigen load. *Eur. J. Immunol.* 34:3525–3533.
23. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature*. 401:708–712.
24. Korber, B.T.M., C. Brander, B.F. Haynes, R. Koup, J.P. Moore, B.D. Walker, and D.I. Watkins, editors. 2005. HIV Molecular Immunology. Los Alamos National Laboratory, Theoretical Biology and Biophysics, Los Alamos, New Mexico. pp. 1–1158.
25. Kiepiela, P., K. Ngumbela, C. Thobakgale, D. Ramduth, I. Honeyborne, E. Moodley, S. Reddy, C. de Pierres, Z. Mncube, N. Mkhwanazi, et al. 2007. CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. *Nat. Med.* 13:46–53.
26. Wilson, N.A., J. Reed, G.S. Napoe, S. Piaskowski, A. Szymanski, J. Furlott, E.J. Gonzalez, L.J. Yant, N.J. Maness, G.E. May, et al. 2006. Vaccine-induced cellular immune responses reduce plasma viral concentrations after repeated low-dose challenge with pathogenic simian immunodeficiency virus SIVmac239. *J. Virol.* 80:5875–5885.
27. Hel, Z., J. Nacsa, E. Tryniszewska, W.P. Tsai, R.W. Parks, D.C. Montefiori, B.K. Felber, J. Tartaglia, G.N. Pavlakis, and G. Franchini. 2002. Containment of simian immunodeficiency virus infection in vaccinated macaques: correlation with the magnitude of virus-specific pre- and postchallenge CD4+ and CD8+ T cell responses. *J. Immunol.* 169:4778–4787.
28. Younes, S.A., B. Yassine-Diab, A.R. Dumont, M.R. Boulassel, Z. Grossman, J.P. Routy, and R.P. Sekaly. 2003. HIV-1 viremia prevents the establishment of interleukin 2–producing HIV-specific memory $CD4^+$ T cells endowed with proliferative capacity. *J. Exp. Med.* 198:1909–1922.
29. Zimmerli, S.C., A. Harari, C. Cellerai, F. Vallelian, P.A. Bart, and G. Pantaleo. 2005. HIV-1-specific IFN-gamma/IL-2-secreting CD8 T cells support CD4-independent proliferation of HIV-1-specific CD8 T cells. *Proc. Natl. Acad. Sci. USA*. 102:7239–7244.
30. Harari, A., F. Vallelian, P.R. Meylan, and G. Pantaleo. 2005. Functional heterogeneity of memory CD4 T cell responses in different conditions of antigen exposure and persistence. *J. Immunol.* 174:1037–1045.
31. Harari, A., S. Petitpierre, F. Vallelian, and G. Pantaleo. 2004. Skewed representation of functionally distinct populations of virus-specific CD4 T cells in HIV-1-infected subjects with progressive disease: changes after antiretroviral therapy. *Blood*. 103:966–972.
32. Heeney, J.L., and S.A. Plotkin. 2006. Immunological correlates of protection from HIV infection and disease. *Nat. Immunol.* 7:1281–1284.
33. Harari, A., V. Dutoit, C. Cellerai, P.A. Bart, R.A. Du Pasquier, and G. Pantaleo. 2006. Functional signatures of protective antiviral T-cell immunity in human virus infections. *Immunol. Rev.* 211:236–254.
34. Precopio, M.L., M.R. Betts, J. Parrino, D.A. Price, E. Gostick, D.R. Ambrozak, T.E. Asher, D.C. Douek, A. Harari, G. Pantaleo, et al. 2007. Immunization with vaccinia virus induces polyfunctional and phenotypically distinctive $CD8^+$ T cell responses. *J. Exp. Med.* 204:1405–1416.

35. Su, L., M. Graf, Y. Zhang, H. von Briesen, H. Xing, J. Kostler, H. Melzl, H. Wolf, Y. Shao, and R. Wagner. 2000. Characterization of a virtually full-length human immunodeficiency virus type 1 genome of a prevalent intersubtype (C/B′) recombinant strain in China. *J. Virol.* 74:11367–11376.
36. Cranenburgh, R.M., J.A. Hanak, S.G. Williams, and D.J. Sherratt. 2001. Escherichia coli strains that allow antibiotic-free plasmid selection and maintenance by repressor titration. *Nucleic Acids Res.* 29:E26.
37. Gomez, C.E., J.L. Najera, V. Jimenez, K. Bieler, J. Wild, L. Kostic, S. Heidari, M. Chen, M.J. Frachette, G. Pantaleo, et al. 2007. Generation and immunogenicity of novel HIV/AIDS vaccine candidates targeting HIV-1 Env/Gag-Pol-Nef antigens of clade C. *Vaccine.* 25:1969–1992.
38. Currier, J.R., E.G. Kuta, E. Turk, L.B. Earhart, L. Loomis-Price, S. Janetzki, G. Ferrari, D.L. Birx, and J.H. Cox. 2002. A panel of MHC class I restricted viral peptides for use as a quality control for vaccine trial ELISPOT assays. *J. Immunol. Methods.* 260:157–172.
39. Kondo, E., Y. Akatsuka, K. Kuzushima, K. Tsujimura, S. Asakura, K. Tajima, Y. Kagami, Y. Kodera, M. Tanimoto, Y. Morishima, and T. Takahashi. 2004. Identification of novel CTL epitopes of CMV-pp65 presented by a variety of HLA alleles. *Blood.* 103:630–638.
40. Burrer, R., D. Salmon-Ceron, S. Richert, G. Pancino, G. Spiridon, S. Haessig, V. Roques, F. Barre-Sinoussi, A.M. Aubertin, and C. Moog. 2001. Immunoglobulin G (IgG) and IgA, but also nonantibody factors, account for in vitro neutralization of human immunodeficiency virus (HIV) type 1 primary isolates by serum and plasma of HIV-infected patients. *J. Virol.* 75:5421–5424.
41. Li, M., F. Gao, J.R. Mascola, L. Stamatatos, V.R. Polonis, M. Koutsoukos, G. Voss, P. Goepfert, P. Gilbert, K.M. Greene, et al. 2005. Human immunodeficiency virus type 1 env clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies. *J. Virol.* 79:10108–10125.
42. Holl, V., S. Hemmerter, R. Burrer, S. Schmidt, A. Bohbot, A.M. Aubertin, and C. Moog. 2004. Involvement of Fc gamma RI (CD64) in the mechanism of HIV-1 inhibition by polyclonal IgG purified from infected patients in cultured monocyte-derived macrophages. *J. Immunol.* 173:6274–6283.