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Expansion and Diversification of Virus-Specific T Cells following Immunization of Human Immunodeficiency Virus Type 1 (HIV-1)-Infected Individuals with a Recombinant Modified Vaccinia Virus Ankara/HIV-1 Gag Vaccine

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Affordable therapeutic strategies that induce sustained control of human immunodeficiency virus type 1 (HIV-1) replication and are tailored to the developing world are urgently needed. Since $CD8^+$ and $CD4^+$ T cells are crucial to HIV-1 control, stimulation of potent cellular responses by therapeutic vaccination might be exploited to reduce antiretroviral drug exposure. However, therapeutic vaccines tested to date have shown modest immunogenicity. In this study, we performed a comprehensive analysis of the changes in virus-specific $CD8^+$ and $CD4^+$ T-cell responses occurring after vaccination of 16 HIV-1-infected individuals with a recombinant modified vaccinia virus Ankara-vectored vaccine expressing the consensus HIV-1 clade A Gag p24/p17 sequences and multiple $CD8^+$ T-cell epitopes during highly active antiretroviral therapy. We observed significant amplification and broadening of $CD8^+$ and $CD4^+$ gamma interferon responses to vaccine-derived epitopes in the vaccinees, without rebound viremia, but not in two unvaccinated controls followed simultaneously. Vaccine-driven $CD8^+$ T-cell expansions were also detected by tetramer reactivity, predominantly in the $CD45RA^-$ $CCR7^+$ or $CD45RA^-$ $CCR7^-$ compartments, and persisted for at least 1 year. Expansion was associated with a marked but transient up-regulation of CD38 and perforin within days of vaccination. Gag-specific $CD8^+$ and $CD4^+$ T-cell proliferation also increased postvaccination. These data suggest that immunization with MVA.HIVA is a feasible strategy to enhance potentially protective T-cell responses in individuals with chronic HIV-1 infection.

Therapeutic immunization in human immunodeficiency virus (HIV) infection is a possible means to achieve viral containment without continuous antiretroviral therapy. Virus-specific cellular immune responses exert control of virus replication in the majority of infected individuals for several years but ultimately fail. When plasma HIV type 1 (HIV-1) RNA falls to undetectable levels under highly active antiretroviral therapy (HAART), the frequencies of virus-specific $CD8^+$ and $CD4^+$ T cells also decline (13, 20, 28), but autovaccination or exposure to autologous virus through intermittent antiretroviral therapy does not lead to improved virus control despite evident restimulation of virus-specific cellular responses (29). Vaccination during HAART has therefore been proposed as a strategy to boost these responses before reexposure to the virus (3). In this context, immunization with live attenuated poxvirus-vectored vaccines or plasmid DNA expressing HIV proteins (8, 11, 16, 19, 21, 39) or inactivated envelope-depleted HIV-1 (32) can elicit new cellular immune responses or amplify preexisting responses to HIV-1. However, it is not known whether responses stimulated by these vaccines will be more effective than those elicited by natural infection, as this has not been fully addressed. Amplification of simian immunodeficiency virus (SIV)-specific cellular immune responses after vaccination of SIV-infected macaques with recombinant poxvirus vaccines expressing SIV proteins was associated with a 10-fold reduction in set-point viremia after antiretroviral therapy was withdrawn which was sustained for several months (38). Data showing that a similar strategy may be effective in humans are scarce (21), however, and the capacity of most of the currently available vaccines to reduce viral load after HAART cessation may be limited.

In the quest for an effective therapeutic strategy, it may be necessary to define immunogenicity in terms of both the magnitude and the quality of induced responses. Recent studies focusing on dissecting the immunological parameters which might distinguish HIV-1-infected long-term nonprogressors from typical progressors have revealed differences in T-cell cytokine secretion profiles, perforin expression, and proliferative capacity (5, 15, 25). Whether these are the cause or consequence of controlled virus replication is uncertain (18). Furthermore, elicitation of HIV-1-specific T cells by prophylactic vaccination failed to prevent disease progression in an individual who subsequently seroconverted, despite the apparently

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favorable functional and phenotypic profile of immune responses after HIV-1 infection had occurred (4). Together, these observations highlight the need for a more comprehensive analysis of vaccine-induced responses in humans in order to identify the candidates which merit evaluation in randomized controlled trials and select the most appropriate immunological endpoints.

Modified vaccinia virus Ankara (MVA) has potential as a vaccine vector: it is safe for use in humans and efficiently stimulates cellular responses to recombinant antigens, particularly after priming with another vaccine (23, 24, 37). MVA.HIVA is a vaccine expressing an immunogen, HIVA, which comprises consensus HIV-1 Gag p24/p17 sequences fused to a multi-cytotoxic T-lymphocyte (CTL) epitope gene (14). It has been evaluated in phase I trials in over 200 HIV-seronegative volunteers in the United Kingdom and east Africa and has an excellent safety profile (I. Cebere, submitted for publication). Recently, we have shown that MVA.HIVA can boost T-cell responses primed by a DNA vaccine encoding the same immunogen (12a, 27). However, data on the safety and immunogenicity of recombinant MVA vaccines in HIV-1-infected individuals are sparse (8, 16). In this study we investigated the effects of intradermal administration of MVA.HIVA to HIV-1-seropositive subjects under fully suppressive HAART. We describe the frequency, breadth, and kinetics of $CD8^+$ and $CD4^+$ T-cell responses amplified by MVA.HIVA immunization in HIV-1-infected individuals. Vaccine-stimulated T cells displayed some of the functional and phenotypic characteristics described in long-term nonprogressors and could thus potentially alter the natural history of infection.

MATERIALS AND METHODS

Study participants. This study was approved by the UK Department of Health Gene Therapy Advisory Committee, the Medicines and Health Products Regulatory Agency, and the local Research Ethics Committee. Eighteen HIV-1-seropositive subjects aged 18 to 60 years (six females) were recruited from local clinics. Inclusion criteria were continuous HAART for at least 12 months, plasma viral RNA of <50 copies/ml (Roche Amplicor assay), CD4 T-cell count of >300 cells/μl, and absence of HIV-related symptoms at screening and 3 months prior to screening. All subjects gave written informed consent. Women were counseled to avoid pregnancy. Blood samples for safety monitoring and immunogenicity studies were obtained at screening (2 to 3 weeks before the first immunization), on the immunization days (days 0 and 28), and 1, 2, 6, 8, 26, and 52 weeks after the first immunization. Subjects were HLA typed by amplification refractory mutation system-PCR using sequence-specific primers.

Vaccine and vaccination schedule. The HIVA gene comprises the consensus HIV-1 clade A Gag p24/p17 sequence fused to a multi-CTL epitope gene and has been described previously (27). MVA.HIVA was produced according to Good Manufacturing Practice by IDT, Germany. Subjects were immunized by intradermal needle injection with two doses of 5×10^7 PFU MVA.HIVA in 0.1 ml normal saline separated by a 4-week interval.

Peptides. Synthetic high-performance liquid chromatography-purified (>80% on mass spectroscopy) peptides (Sigma-Genosys) based on the sequence of the HIVA immunogen comprised (i) 90 Gag p24/p17 15-mer peptides overlapping by 11 amino acids and (ii) 23 Gag/Pol/Nef/Env epitope peptides (optimal 9- to 11-mers). The overlapping Gag peptides were used in pools of 9 to 10 peptides in a matrix array in gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assays. In carboxyfluorescein succinimidyl ester (CFSE) assays, four sequential pools of 22 to 23 peptides (HIVA pools 1 to 4′) or one pool of 90 peptides (pool 90) was used, as blood sample volumes at each bleed were limited to 50 ml. Pools 1, 2, 3, and 4 represented Gag $p24^{1–100}$, $p24^{90–188}$, $p24^{178–231}$ plus $p17^{1–48}$, and $p17^{38–132}$, respectively. The epitope peptides were tested either as a pool (9′) or individually. Pool 8′ comprised known $CD8^+$ T-cell epitopes derived from cytomegalovirus, Epstein-Barr virus (EBV), and influenza virus proteins and was used as an additional positive control to phytohemagglutinin (PHA).

IFN-γ ELISPOT assay. The IFN-γ ELISPOT assay was performed as described previously (27). Briefly, freshly isolated peripheral blood mononuclear cells (PBMC; 1×10^5/well) were incubated in duplicate with (i) overlapping Gag peptide matrix pools, (ii) pool 9 peptides, (iii) pool 8 (positive control) peptides, (iv) medium alone (in quadruplicate), or (v) PHA (5 μg/ml; Murex, United Kingdom). The final concentration of each peptide was 4 μg/ml. $CD8^+$-cell depletions were achieved with MACS beads (Miltenyi-Biotec, United Kingdom) according to the manufacturer's instructions. Spot-forming units (SFU) were quantified with an automated ELISPOT plate reader (AID Systems, Germany) by an observer blinded to the subjects' immunization status. The frequencies of HIV-1-specific IFN-γ-releasing cells were expressed as IFN-γ SFU/10^6 PBMC, after subtraction of the number of SFU in negative control wells. Criteria for accepting assays were <50 SFU/10^6 PBMC in unstimulated wells and saturation with color development reagent in PHA-stimulated wells. The breadth of the Gag-specific response was defined as the number of matrix pools yielding a response (>100 SFU/10^6 PBMC for this analysis) divided by 2, since each peptide was included twice in the matrix. Therefore, a response to all pools was scored as 9.5.

Tetramer and phenotype analysis. HLA-A*0201, -A3, -B7, -B8, -B*2705, and -B35 peptide tetrameric complexes were synthesized as described previously (10). Tetramer-reactive cells were detected by incubating aliquots of fresh or thawed cryopreserved PBMC (1×10^6) with fluorochrome (phycoerythrin [Sigma])-labeled tetramers at 37°C for 15 min, followed by a further 20-min incubation at room temperature with antibodies to CD8, CD38, CD45RA, and CCR7. Intracellular perforin staining was performed after permeabilization. Samples were washed, fixed, and analyzed by flow cytometry. Typically, 200,000 events were acquired in the lymphocyte gate.

Ex vivo proliferation assay. Freshly isolated PBMC were labeled with a predetermined concentration of CFSE (0.8 μM). The reaction was quenched with RPMI 1640 supplemented with 10% AB human serum, L-glutamine, and penicillin-streptomycin (H10 medium), and cells were washed twice before resuspending in H10 at a density of 2×10^6/ml. Cells were cultured in 96-well round-bottomed plates (2×10^5/well in duplicate) with medium alone, HIVA peptide pools 1 to 4, or pool 90 (final concentration, 2 μg/ml) or staphylococcal enterotoxin B (1 μg/ml) for 6 days at 37°C. Cells were then harvested, washed, stained with CD8-phycoerythrin, CD4-peridinin chlorophyll *a*, or CD3-allophycocyanin and fixed. Approximately 5×10^4 events in the $CD3^+$ $CD4^+$ or $CD3^+$ $CD8^+$ lymphocyte gates were acquired on a FACSCalibur four-color flow cytometer using CellQuest software and subsequently analyzed using ModFit LT software. Antigen-specific proliferation was expressed either as the percentage of $CFSE^{lo}$ cells in peptide-stimulated samples minus the percentage of $CFSE^{lo}$ cells in unstimulated samples (single time point analyses) or as the cell division index (the percent peptide-stimulated $CFSE^{lo}$ cells divided by the percent unstimulated $CFSE^{lo}$ cells; longitudinal analyses). A positive response to peptides was defined as one which was at least three times the response in the unstimulated sample.

Statistical analysis. Results were expressed as the median plus range or interquartile range. Paired samples (pre- and postimmunization) were analyzed by applying the Wilcoxon signed-rank test using GraphPad Prism software.

RESULTS

MVA.HIVA immunizations were safe in HAART-treated patients with chronic HIV-1 infection. Sixteen subjects (five females) volunteered to receive two doses of MVA.HIVA, 5×10^7 PFU, by intradermal needle injection 4 weeks apart. Eight subjects had received a DNA vaccine which expresses the same immunogen, pTHr.HIVA, 2 years earlier (11). All subjects completed the immunization schedule according to the study protocol and remain under long-term follow-up. Two additional subjects were recruited as controls (one female) and were venesected in parallel with the vaccinees. Nine subjects had most likely acquired HIV-1 infection in Africa or Asia and nine acquired infection within Europe or the Americas. At baseline, the median CD4 T-cell count was 530 cells/μl (range, 300 to 810), the pre-HAART CD4 T-cell nadir was 165 cells/μl (range, 10 to 240), and volunteers had been taking HAART for a median 50 months (range, 12 to 100) (Table 1). The patients' HAART regimens were continued throughout the study pe-

TABLE 1. Clinical characteristics of volunteers

Treatment group and subject no.	Age (yr)	CD4 T-cell count (cells/μl)	CD4 nadir	HAART (mo)	HLA alleles	
					A and B	DRB1
MVA/HIVA only						
011	37	650	240	19	A30, 74 B*5802/06	DRB1*15, *11
012	34	720	140	62	A1, 3 B7, 8	DRB1*03, *13
013	41	600	100	50	A*0201, 1 B8, 18	DRB1*03, *04
014	48	380	200	12	A*03, 11 B42, 53	DRB1*15, *1001
015	32	390	10	61	A*0201, 3 B*2705, 53	DRB1*1001, *1302
016	44	310	160	42	A2 B18, B*0710	DRB1*04
018	25	430	200	17	A24, *3401 B40, 56	DRB1*04, *11
019	25	670	230	19	A2, 24 B35, 57	DRB1*08
021	58	420	240	12	A1, *0201 B8, 44	DRB1*13, *11
022	38	300	160	19	A29, 30 B57, 18	DRB1*11, *13
MVA.HIVA 2 yrs after pTHr.HIVA						
001	48	810	240	67	A2, *03 B35, *44	DRB1*13
002	50	770	20	98	A*0102, *33 B*44, *5802	DRB1*12, *13
004	54	800	40	77	A1, *03 B35, 37	DRB1*01, *15
005	44	460	170	55	A2, 29 B*45, *5802	DRB1*12, *13
006	34	520	10	90	A2 B15, *44	DRB1*04
007	35	540	190	51	A*0201, 03 B*35, *44	DRB1*01, *07
009	40	700	180	100	A3, 11 B7	DRB1*15
010	41	510	140	38	A*01, 1 B18, 35	DRB1*03, *09

riod, and plasma HIV-1 RNA remained below 50 copies/ml in all subjects. The vaccine was well tolerated, and no serious adverse events were observed. Detailed safety and tolerability data are reported elsewhere (P. Williams, unpublished data).

MVA.HIVA immunization amplifies and broadens HIV-1-specific $CD8^+$ and $CD4^+$ T-cell IFN-γ responses. Prevaccination responses (at screening and day zero visits) to HIVA Gag p24/p17 15-mer peptides overlapping by 11 amino acids (HIVA OLP) were detected by IFN-γ ELISPOT in all 18 volunteers. The total Gag-specific response was defined as the sum of all Gag peptide-specific responses divided by 2, since each peptide was duplicated in the matrix array. The median frequency at baseline was 1,301 SFU/10^6 PBMC (range, 82 to 4,050) (Fig. 1A). $CD8^+$-cell depletions abrogated >50% of the response in 15/18 volunteers, indicating that these baseline Gag-specific responses were mediated predominantly by $CD8^+$ cells (Fig. 1A). Responses to Nef, Pol, and Env optimal epitope peptides, which comprised 17/23 $CD8^+$ T-cell epitopes in the epitope string, were observed in only three subjects. This likely reflected both the genetic background of the volunteers, as not all had HLA class I haplotypes corresponding to the known restricting elements of these epitopes (14), and the dominance of responses to Gag over non-Gag epitopes in chronic treated infections (1) (Fig. 1C and Table 1).

After one or two MVA.HIVA immunizations, the total Gag-specific response increased in all 16 vaccinees, by ≥2-fold or >1,000 SFU in 12 vaccinees. Overall, responses were significantly greater at all postvaccination time points than at day zero (medians at day 0, week 2, and week 8, 1,508, 2,390, and 2,751 SFU/10^6 PBMC, respectively; $P = 0.0005$ and 0.001) (Fig. 1A). The peak response occurred after the first dose in 6 vaccinees, while in 10 subjects a further increment was seen after the second dose. When the MVA.HIVA-only group was compared with the DNA-MVA.HIVA group, peak Gag-specific responses were similar; the only difference between the two subgroups was that the peak response was attained after the first MVA.HIVA dose in four subjects who had had prior DNA vaccination compared with two who had received MVA.HIVA alone. T-cell frequencies were still significantly elevated 6 months after the first immunization (median, 1,998 SFU/10^6 PBMC; $P = 0.004$) by >2-fold in 4/15 vaccinees who have reached this time point.

As $CD8^+$-cell-depleted ELISPOT assays were performed in parallel, it was evident that Gag-specific $CD4^+$ T cells were also augmented by MVA.HIVA immunization in 13/15 vaccinees but were unaffected in 2 vaccinees. One subject (007) was excluded from this analysis because of high background responses 7 and 14 days after the first vaccination. The median total $CD8^+$-cell-depleted Gag-specific responses at day 0 and weeks 2, 6, and 27 were 195, 435, 346, and 404 SFU/10^6 PBMC, respectively ($P = 0.015$, 0.027, and 0.058) (Fig. 1B). $CD4^+$ T cells contributed most of the postimmunization Gag-specific increment in five vaccinees. Subgroup analysis did not show significant differences in the magnitude of pre- and post-MVA.HIVA responses in volunteers who had received MVA.HIVA alone or prior DNA immunization.

MVA.HIVA immunization amplified not only the magnitude but also the breadth of Gag-specific $CD8^+$ or $CD4^+$ T-cell responses. The number of Gag OLP pools eliciting a response greater than 100 SFU/10^6 PBMC increased after immunization in 15/16 vaccinees, with significantly more pools being targeted at the peak of the response than at baseline (day zero and peak postvaccination; medians, five and seven pools, respectively; $P < 0.0001$) (Fig. 1C). Analysis of responses to the peptide matrix revealed the epitopic regions of the p24 and p17 proteins targeted by each subject. These were confirmed in subsequent assays with individual 15-mers and predefined CTL epitope peptides within these 15-mers. Boosting of responses to one or more peptides by at least threefold was observed in 12/16 vaccinees (data not shown). The maximum increment in

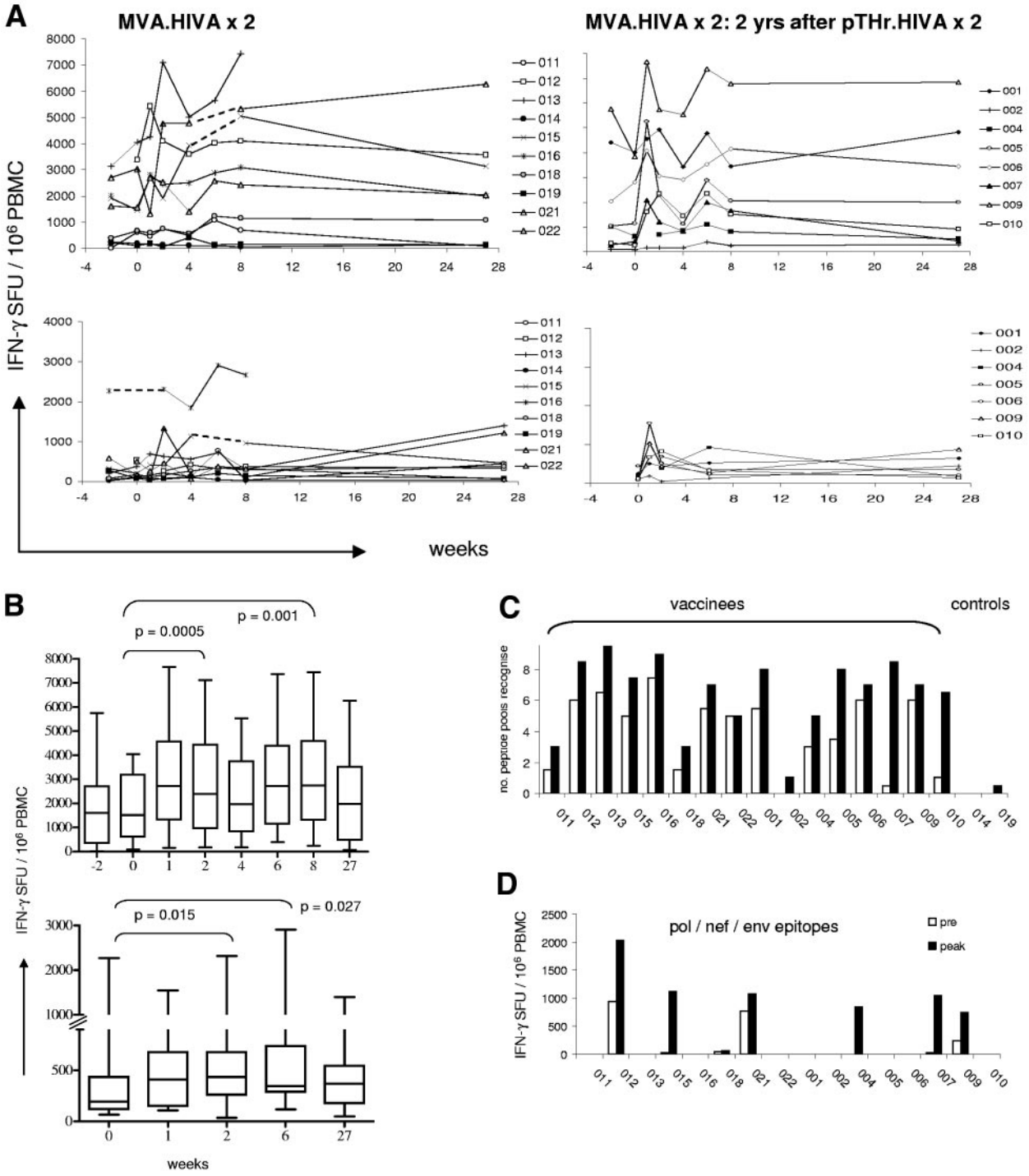

FIG. 1. Responses to overlapping HIVA Gag 24/p17 15-mer and non-Gag epitope peptides obtained in ex vivo IFN-γ ELISPOT assays. (A) Total Gag-specific response (sum of peptide matrix pool responses divided by 2) obtained in assays with fresh undepleted (top panels) or $CD8^+$-cell-depleted (bottom panels) PBMC from 16 vaccinees and 2 unvaccinated control subjects (014 and 019) (solid black symbols in left panels) at the time points indicated. Immunizations were given at days 0 and 28. Negative control values have been subtracted. Broken lines indicate that no sample was available for the intervening time point. (B) Box plots (interquartile range with median indicated by horizontal line) of total Gag responses of the 16 vaccinees in assays with undepleted (top) or CD8-depleted (bottom) PBMC at the time points indicated. (C) Breadth of Gag-specific responses in IFN-γ ELISPOT assays with undepleted PBMC before (white bars) and at the peak of the response (black bars) after one or two MVA.HIVA immunizations or, for the control subjects, the highest response seen at any time after day zero. (D) Preimmunization (white bars) and peak postimmunization (black bars) responses in IFN-γ ELISPOT assays with optimal CTL epitope peptides derived from Pol, Nef, and Env expressed in the epitope string (14).

TABLE 2. Peptides containing undefined $CD8^+$ and $CD4^+$ T-cell epitopes in Gag p24/p17 recognized by MVA.HIVA vaccine responders[a]

Peptide and sequence	Responder subject no.	Fold increase post-MVA.HIVA
$CD8^+$ peptides		
KIRLRPGGKKKYRLK	012	2.6
MTSNPPIPVGDIYKR	022	6.7
VRMYSPVSILDIRQG	022	3.2
KDTKEALDKIEEIQN	001	4.3
RDYVDRFFKTLRAEQ	005	23
TETLLVQNANPDCKS	005	18
GGKLDAWEKIRLRPG	005	21
ERFALNPSLLE	005	18
$CD4^+$ peptide sequence		
PQDLNMMLNIVGGHQA	016	3.4
HQAAMQMLKDTINEE	016	3.6
GGKLDAWEKIRLRPG	005	2

[a] Postvaccination responses were defined as ≥2-fold above preimmunization values. Peptides containing published epitopes were included if they were not known to be restricted by any of the HLA alleles expressed by the vaccine responder.

a response to a single peptide after vaccination was 1,830 SFU. Five subjects generated $CD8^+$ or $CD4^+$ T-cell responses to at least 10 peptides for which no epitopes have been defined or have been defined as restricted by HLA alleles not expressed by the vaccinees (Table 2). Further analysis of these responses is under way (B. Ondondo, unpublished data). Responses to Pol, Nef, and Env epitopes in the $CD8^+$ T-cell epitope string were amplified or induced in 6/16 vaccinees (Fig. 1D).

The frequencies of T cells specific for Gag p24/p17 OLP and pool 9 peptides were determined in parallel in two unvaccinated control subjects who agreed to follow a bleed schedule identical to that of the vaccinees. Serendipitously, these two individuals had lower Gag OLP responses at baseline than the majority of vaccinees. However, in contrast to subject 011, who had the lowest baseline level of Gag-specific T cells and who developed the smallest response to MVA.HIVA (>1,000 SFU/10^6 PBMC at its peak), responses in the controls remained stable (subject 014) or increased to a maximum of 417 SFU/10^6 PBMC at a single time point (subject 019) (Fig. 1A).

Tetramer staining reveals long-lived vaccine-driven $CD8^+$ T-cell expansions, predominantly in the $CCR7^-$ $CR45RA^-$ and $CCR7^+$ $CD45RA^-$ compartments. We next used a panel of 13 fluorochrome-conjugated HLA class I (HLA-A*0201, -A3, -B7, -B8, or -B*2705) tetrameric complexes to quantify antigen-specific $CD8^+$ T cells specific for vaccine-, HIV-1 non-vaccine-, and EBV-derived epitopes. Assays were therefore confined to vaccinees with these HLA alleles (subjects 012, 013, 015, 021, 001, 004, 006, 007, and 009). Vaccine-driven $CD8^+$ T-cell expansions of at least twofold and with specificity for eight different epitopes were seen in 7/9 vaccinees. The peak responses detected by tetramer reactivity were seen after the second immunization. In contrast, $CD8^+$ T cells specific for non-vaccine (EBV- or other HIV-1-derived) epitopes varied minimally from baseline values, by a median of 1.3-fold (Fig. 2A and B). The vaccine-induced expansions were maintained at near-peak levels for 12 months or were 10-fold higher than at baseline (subject 015, HLA-B*2705 p24-specific T cells) in 4/4 subjects who have reached this time point. Of note, although the functional responses to the same epitopes, indicated by the IFN-γ ELISPOT assay, had declined by this time, some remained higher than preimmunization values (data not shown).

Analysis of the expression of differentiation and memory markers CD45RA and CCR7 in tetramer-positive cells before and 6 months after MVA.HIVA immunization (6, 17) revealed that expansion was predominantly in the $CCR7^+$ $CD45RA^-$ compartment in 6/7 subjects, but the $CCR7^-$ $CD45RA^-$ compartment was also augmented considerably. Some expansion of the $CCR7^-$ $CD45RA^+$ and $CCR7^+$ $CD45RA^+$ compartments also occurred, but to a much lesser extent (Fig. 2C and Table 3).

MVA.HIVA immunization is followed by transient $CD8^+$ T-cell activation and increased perforin expression without rebound viremia. In order to ascertain whether vaccine-driven $CD8^+$ T-cell expansions were distinguishable from cell populations which were not boosted by MVA.HIVA immunization, we examined the expression of the activation marker CD38 on tetramer-positive cells. We observed marked upregulation of CD38 in tetramer-positive cells 1 to 2 weeks after MVA.HIVA vaccination: in 8/9 subjects in whom tetramer staining was performed, the percentage of tetramer-positive cells expressing CD38 increased by at least fivefold in one or more tetramer-positive populations. The kinetics of CD38 expression varied between T cells of different specificities within each individual, with a peak at day 7 in five vaccinees. In all subjects, CD38 expression had returned to baseline by day 28, but further activation was observed after the second immunization in four vaccinees (Fig. 3A and B). CD38 expression in the total $CD8^+$ T-cell population (Fig. 3B) and in $CD8^+$ T cells specific for a non-HIVA Nef epitope (subject 015) (Fig. 3A) or EBV-derived epitopes (subject 012) (data not shown) was not significantly up-regulated at any time point analyzed. Viral loads in plasma samples taken at each bleed were consistently below the limit of detection (<50 copies/ml). At baseline, tetramer-positive cells expressed low levels of perforin, consistent with a previous study of chronically infected individuals (2). However, after MVA.HIVA immunization, $CD8^+$ T-cell activation was accompanied by an increase of at least twofold in the percentage of tetramer-positive cells expressing perforin (Fig. 3C; representative of four subjects), while the proportion of perforin-expressing cells in the total $CD8^+$ T-cell population remained stable over the same time (data not shown).

$CD8^+$ and $CD4^+$ T-cell proliferative responses to HIV-1 Gag are enhanced by vaccination. It is not known whether induction of virus-specific lymphoproliferative responses by vaccination can enhance virological control, but this is suggested by clinical trials and by inference from studies of individuals who maintain low virus loads without therapy (5, 21, 39, 42). We therefore analyzed the capacity of $CD8^+$ and $CD4^+$ T cells to proliferate in response to overlapping Gag peptides in a 6-day CFSE assay. Analysis of the MVA.HIVA-alone group was restricted to postvaccination samples in five consecutive vaccinees and one control because of limited sample availability. Gag-specific $CD3^+$ $CD8^+$ $CFSE^{lo}$ cells ranging from 1.9 to 52.3% were detected in these five vaccinees, and $CD3^+$ $CD4^+$ $CFSE^{lo}$ cells were detected in three of these individuals postimmunization (Fig. 4A). Of note, $CD4^+$ T-cell proliferative responses were greatest in subject 016, who maintained the highest level of HIV-1 Gag-specific IFN-γ-secreting $CD4^+$ T

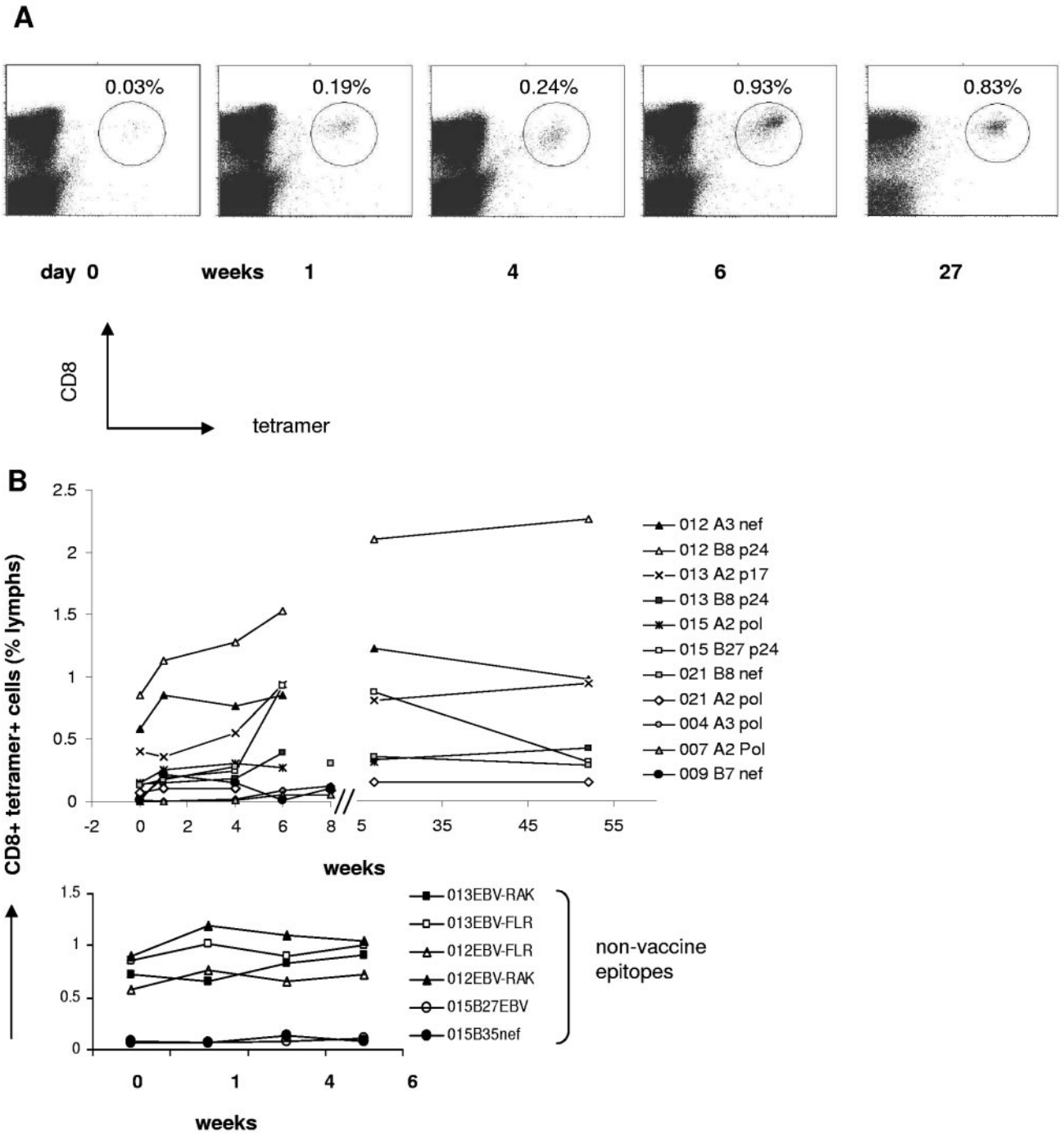

FIG. 2. Postimmunization expansion of $CD8^+$ T cells specific for HIV-1 epitopes in MVA.HIVA, identified by tetramer staining. (A) Representative profile showing evolution of the HLA-B*2705/p24-specific response of subject 015 at the times indicated. Ex vivo PBMC were stained with an HLA-B*2705 tetramer refolded with the KRWIILGLNK peptide. Dot plots show gated lymphocytes identified by forward and side scatter. (B) Top panel: frequencies of HIV-1-specific $CD8^+$ T cells in seven vaccinees determined by staining with various tetramers at the times indicated. Tetramers were refolded with the following peptides: A2 p17, SLYNTVATL; A2 Pol, ILKEPVHGV; A3 Pol, AIFQSSMTK; A3 Nef, QVPLR PMTYK; B7 Nef, TPGPGVRYPL; B8 p24, GEIYKRWII; B8 Nef, FLKEKGGL; B27 p24, KRWIILGLNK. Bottom panel: frequencies of $CD8^+$ T cells specific for non-vaccine-derived epitopes from EBV proteins (subjects 012, 013, and 015) or HLA-B35 Nef (GPGVRYPLTF) (subject 015) at corresponding time points. (C) Representative plots (subject 015) showing expression in tetramer-reactive cells (top panels) of CD45RA and CCR7 before and 6 and 27 weeks after the first MVA.HIVA immunization. Bottom panels show profiles obtained by gating on total $CD8^{bright}$ lymphocytes. The fold change in each compartment within tetramer-positive populations detected in seven donors is shown in Table 3.

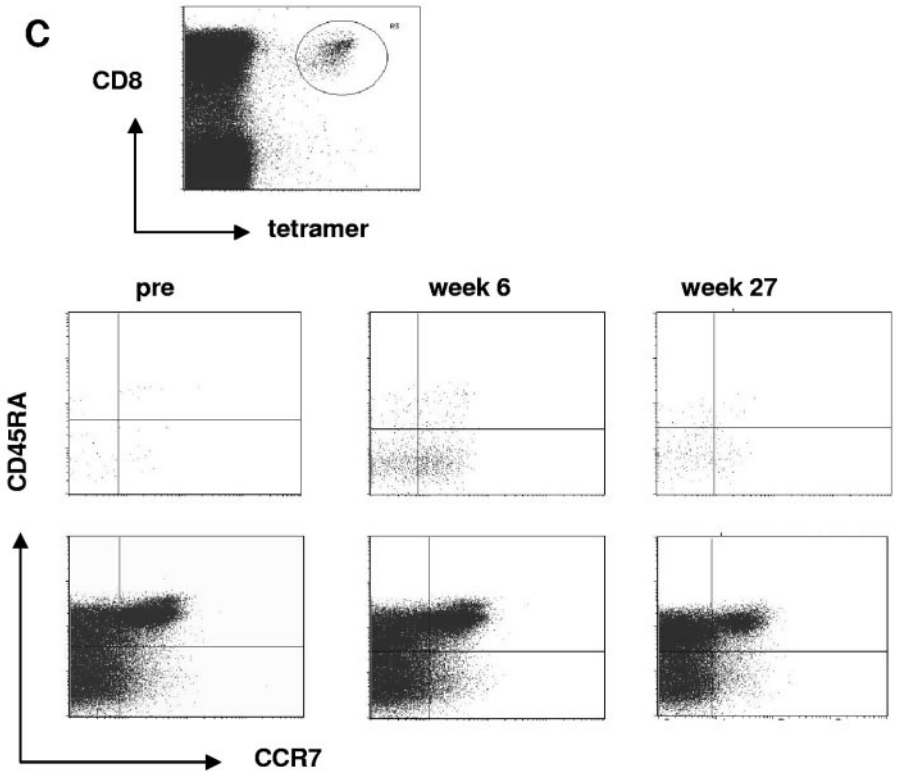

FIG. 2—*Continued.*

cells throughout the study. A $CD4^+$ T-cell proliferative response to one of the peptide pools was just detectable in one of the control subjects, 019, at the corresponding time point. Proliferation was assessed immediately before and at three post-MVA.HIVA immunization time points in the DNA/MVA group (Fig. 4B and C). A significant $CD8^+$ T-cell proliferative response to Gag peptides, defined as a cell division index of >3, developed in three vaccinees (001, 004, and 010), while in three with preexisting positive responses, these increased at least fourfold after vaccination (005, 006, and 009). $CD4^+$ T-cell proliferative responses were induced or amplified in 001, 004, 005, 006, 007, and 010. Subject 002 did not show increased responses, and 009 is still under evaluation.

TABLE 3. Relative expansion of $CD8^+$ T-cell memory compartments in tetramer-positive populations 6 months after MVA.HIVA immunization[a]

Subject (HLA) and response	Fold change from baseline			
	$CD45RA^+$ $CCR7^-$	$CD45RA^+$ $CCR7^+$	$CD45RA^-$ $CCR7^+$	$CD45RA^-$ $CCR7^-$
012[b] (B7), Nef	0	0.53	12	7.8
012[b] (B8), p24	5	2.46	17.8	9.2
012[b] (A3), Nef	1.98	1.2	14.1	5.6
013 (B8), p24	0	3.4	18.6	10.2
015 (B27), p24	2.2	10.4	11.4	14.6
021 (B8), Nef	2.2	0.14	7.3	6.3
004 (A3), Pol	3.68	1.54	18.15	6.5

[a] Compartments within tetramer-positive populations were defined as shown in Fig. 2C. Fold changes from preimmunization values were calculated after adjusting for the number of $CD8^+$ lymphocytes acquired (typically 2×10^5).

[b] Postvaccination analysis for subject 012 was performed on the 12-month sample.

DISCUSSION

This study shows that a therapeutic immunization strategy employing intradermal administration of MVA.HIVA to chronically HIV-1-infected individuals under HAART suppression is able to elicit broad, durable virus-specific $CD8^+$ and $CD4^+$ T-cell responses. The immunogenicity of recombinant MVA that we observed is particularly impressive when considering the following: (i) the study cohort comprised patients with more advanced disease (median pre-HAART CD4 T-cell nadir, 180 cells/μl) than those included in previously reported studies (19, 21, 32, 39, 40); (ii) the dose of MVA was modest compared with previous human and nonhuman primate studies (8, 16, 33, 41); and (iii) the sequence of the immunogen is based on the clade A consensus sequence (14), whereas the majority of vaccinees were known to be infected with other subtypes (data not shown). Furthermore, HIV-1 infection per se did not appear to inhibit the generation of responses to MVA.HIVA despite the potential for immune interference (36). Indeed, the rapidity of T-cell expansion after immunization and the magnitude of the peak responses, which were 10- to 100-fold greater than responses in HIV-uninfected individuals given the same vaccine (12a), suggested that the vaccine was boosting responses primed by HIV-1 itself. As patients were maintained on HAART after immunization, plasma HIV-1 RNA was undetectable in all vaccinees at all

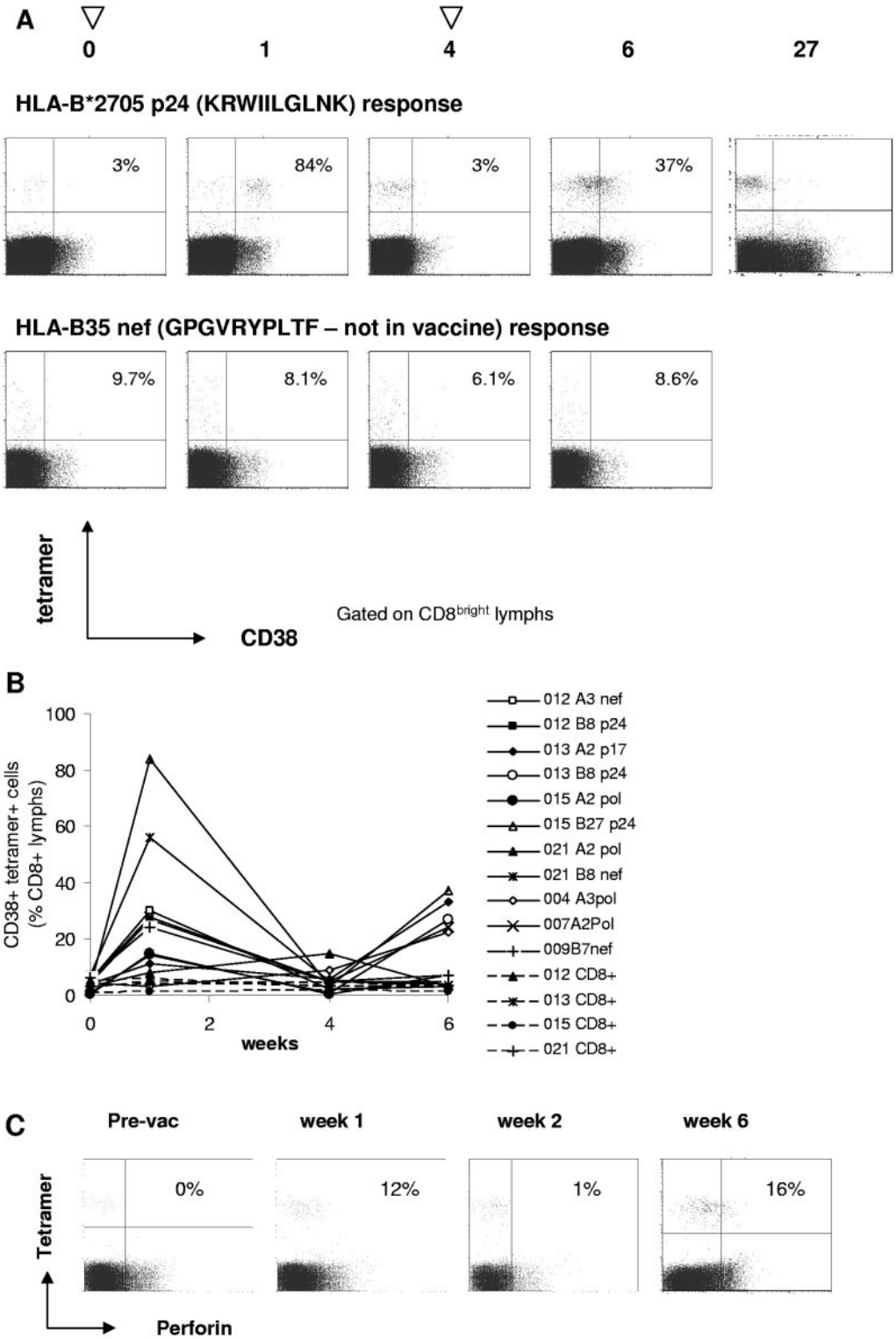

FIG. 3. Up-regulation of CD38 on $CD8^+$ T cells specific for vaccine-encoded epitopes. (A) Top panels: CD38 expression in KRWIILGLNK-specific T cells (gated on tetramer-positive cells as shown in Fig. 2C) from subject 015. Bottom panels: Nef-specific (epitope not in the vaccine) tetramer-positive cells from this donor showed stable levels of CD38 expression over the same period. Triangles indicate immunization times. (B) Kinetics of CD38 expression in tetramer-positive cells with different specificities (solid lines) (see Fig. 2B for specificities) and total $CD8^+$ T cells (broken lines) obtained from seven donors before and after MVA.HIVA immunization. (C) Kinetics of perforin expression on tetramer-positive cells is similar to that of CD38. The percentage of HIV-1-specific tetramer-positive cells expressing perforin pre- and post-MVA.HIVA immunization are shown for subject 015 (representative of four vaccinees). The percentage of total $CD8^+$ cells expressing perforin remained stable over this time (6% [not shown]).

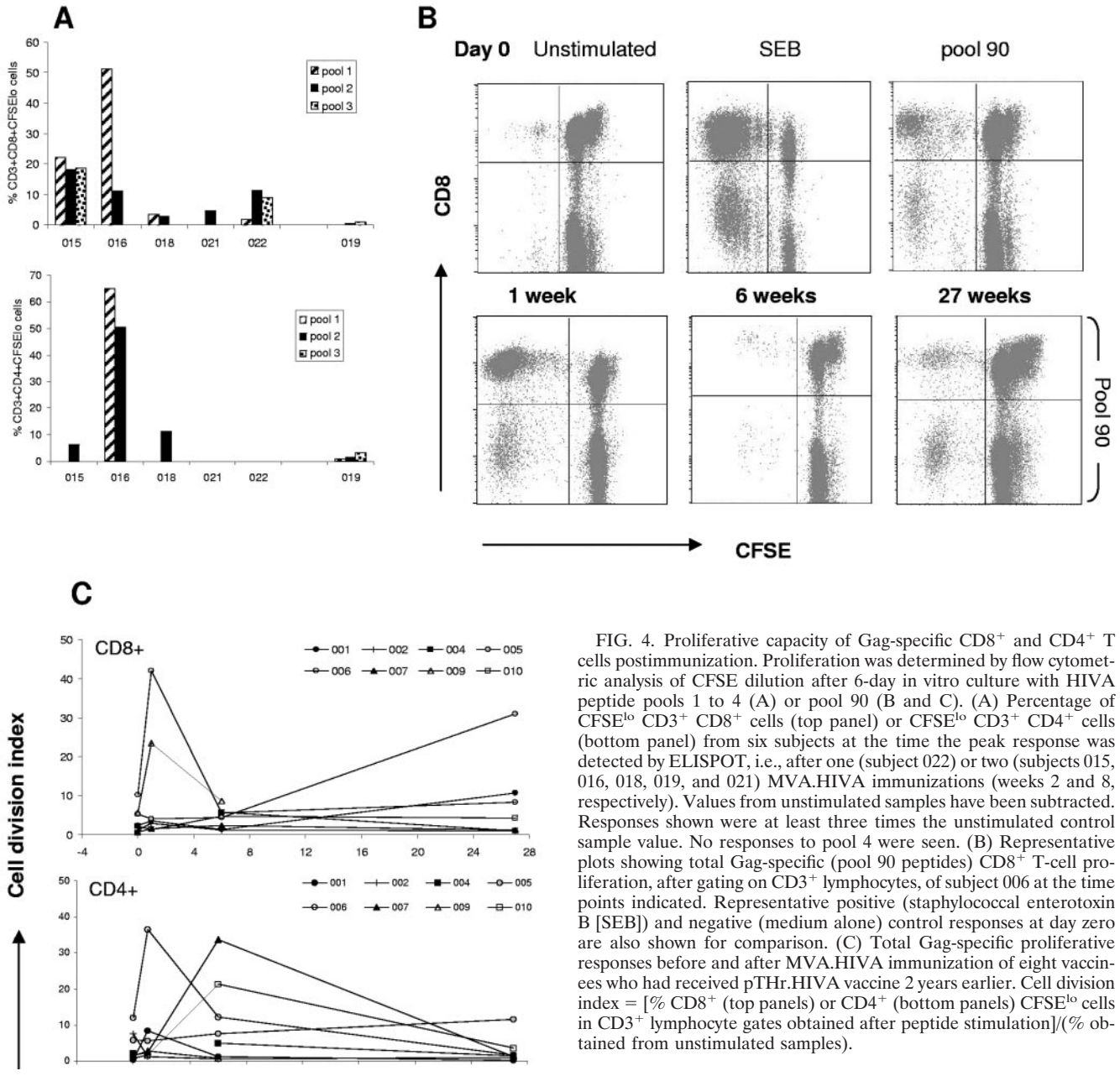

FIG. 4. Proliferative capacity of Gag-specific $CD8^+$ and $CD4^+$ T cells postimmunization. Proliferation was determined by flow cytometric analysis of CFSE dilution after 6-day in vitro culture with HIVA peptide pools 1 to 4 (A) or pool 90 (B and C). (A) Percentage of $CFSE^{lo}$ $CD3^+$ $CD8^+$ cells (top panel) or $CFSE^{lo}$ $CD3^+$ $CD4^+$ cells (bottom panel) from six subjects at the time the peak response was detected by ELISPOT, i.e., after one (subject 022) or two (subjects 015, 016, 018, 019, and 021) MVA.HIVA immunizations (weeks 2 and 8, respectively). Values from unstimulated samples have been subtracted. Responses shown were at least three times the unstimulated control sample value. No responses to pool 4 were seen. (B) Representative plots showing total Gag-specific (pool 90 peptides) $CD8^+$ T-cell proliferation, after gating on $CD3^+$ lymphocytes, of subject 006 at the time points indicated. Representative positive (staphylococcal enterotoxin B [SEB]) and negative (medium alone) control responses at day zero are also shown for comparison. (C) Total Gag-specific proliferative responses before and after MVA.HIVA immunization of eight vaccinees who had received pTHr.HIVA vaccine 2 years earlier. Cell division index = [% $CD8^+$ (top panels) or $CD4^+$ (bottom panels) $CFSE^{lo}$ cells in $CD3^+$ lymphocyte gates obtained after peptide stimulation]/(% obtained from unstimulated samples).

time points sampled, and we do not yet know what impact the vaccine-stimulated responses might have on viral rebound. The relatively limited effects of other therapeutic vaccination protocols have raised questions regarding the number of immunizations, the optimal time to stop therapy, and the most appropriate immunological and virological end points (7, 21, 39). For this reason, we performed a detailed qualitative and kinetic analysis of the T-cell responses stimulated by MVA.HIVA in order to better inform the design of an analytical therapy interruption study.

The amplification of both Gag-specific $CD8^+$ and $CD4^+$ T cells that we observed following MVA.HIVA vaccination contrasts with the findings of another therapeutic study with recombinant MVA-Nef, where little or no effect on $CD8^+$ T-cell responses was observed (8). This could be due to several factors, including the dose and route of immunization and the assay used to measure $CD8^+$ T-cell responses, or it may reflect differences in the capacity of recombinant MVA vaccines to boost Gag- versus Nef-specific responses in chronically infected individuals. Spontaneous variations in individual patients' preexisting HIV-1-specific responses cannot be completely ruled out without more control subjects, but we believe this is unlikely for several reasons. First, the kinetics of these responses, with ex vivo Gag-specific IFN-γ-secreting T-cell responses peaking at 1 to 2 weeks after each immunization, are consistent with data from other recombinant MVA vaccine studies (23, 24). Second, minimal variation was seen in two unvaccinated control subjects monitored simultaneously. Third,

we compared the peak responses to MVA.HIVA with those seen after immunization of 10 HAART-treated patients with a DNA vaccine expressing the same immunogen (pTHr.HIVA) in a previous study (11). Ex vivo Gag-specific IFN-γ responses immediately prior to pTHr.HIVA and to MVA.HIVA (>2 years after the DNA vaccinations in subjects 001 to 010) were not significantly different (medians, 831 and 1,508 SFU/10^6 PBMC, respectively; $P = 0.13$), and responses 28 days after the second dose of each vaccine (day 56 for MVA.HIVA and day 49 for pTHr.HIVA) were much greater in the MVA.HIVA arm (medians, 380 and 2,751; $P = 0.005$).

Of note, $CD4^+$ and $CD8^+$ T-cell responses post-MVA.HIVA vaccination were increased not only in magnitude but also in breadth, with targeting of $CD8^+$ T-cell epitopes in Nef, Pol, and Env proteins which were expressed in the epitope string, together with conserved regions of the Gag protein which are known to be frequently recognized by diverse ethnic populations (12), and of previously unrecognized Gag epitopes. This indicates that it is possible to boost responses to subdominant T-cell epitopes with a recombinant MVA vaccine. As the volunteers in this study had all progressed to AIDS or had developed low CD4 T-cell counts prior to the study, it is likely that they would harbor $CD8^+$ T-cell escape mutants which could emerge if antiretroviral therapy were interrupted. For a therapeutic vaccine to be effective, it will be necessary to elicit T cells which can suppress the autologous virus variants, and this may require T cells with broad specificity and clonality (30, 34, 35). Our data suggest that it may be possible to refocus HIV-1-specific T-cell responses by vaccination, even in chronically infected individuals with diverse virus subtypes and genetic backgrounds.

By contrast with the transient responses detected by IFN-γ ELISPOT, tetramer studies showed that MVA.HIVA-driven $CD8^+$ T-cell expansions, which involved different memory compartments, were more durable. Up-regulation of the activation marker CD38 within days of vaccination predicted the development of sustained $CD8^+$ T-cell responses. Low frequencies of activated virus-specific $CD8^+$ T cells at this time point predicted little or no expansion of these cells at subsequent time points. This was evident in both intra- and interindividual analyses comparing $CD8^+$ T cells specific for different vaccine-encoded epitopes and for epitopes not included in the vaccine. Expression of CD38 on $CD8^+$ T cells is a marker of viral replication in acute or untreated chronically infected individuals (26), but it is highly unlikely that the changes we observed were due to enhanced viral replication, since plasma HIV-1 RNA was undetectable in the vaccinees throughout the study. The effect of MVA.HIVA immunization on perforin expression in $CD8^+$ T cells may also be important, since a previous study showed that vaccine-stimulated $CD8^+$ perforin-expressing T cells were positively correlated with viral suppression (22). However, the rapid declines in perforin-expressing and, to a lesser extent, IFN-γ-secreting $CD8^+$ T cells suggest that these cells do not remain fully functional in the absence of the antigenic stimulation provided by vaccination, or they may indicate activation-induced apoptosis. The capacity of these long-lived vaccine-driven $CD8^+$ T cells to differentiate rapidly into fully functional effectors may be a critical factor in the control of virus rebound after withdrawal of antiretroviral therapy. We observed predominant expansion in the $CCR7^+$ $CD45RA^-$ compartment, which has proliferative potential (6), and obtained direct evidence for vaccine-driven $CD8^+$ T-cell proliferation in the majority of vaccinees. Together, these observations suggest that our vaccination strategy could alter the composition of the HIV-1-specific $CD8^+$ T-cell memory pool. A prophylactic vaccination study in macaques suggested that the kinetics of viral replication in relation to $CD8^+$ T-cell division and differentiation may be crucial to preventing established infection (9). The magnitude or quality of cellular responses required to prevent infection may be different from that needed to suppress viral replication during chronic infection, but this question will need to be addressed in future studies involving vaccination followed by analytical therapy interruption.

To date, there have been no studies comparing the immunogenicity of different virus-vectored vaccines (e.g., MVA, canary pox virus, and NYVAC) in HIV-1-infected individuals. Recombinant canary pox virus (ALVAC)-vectored vaccines have undergone more extensive evaluation in humans than other poxvirus-based vaccines, and immunogenicity and preliminary efficacy data in HIV-1-infected individuals are encouraging, with evidence of a positive correlation between virological control and vaccine-stimulated $CD4^+$-cell proliferative responses (21, 39). However, the response rate observed in these studies using the criterion of lymphoproliferation (50 to 60%) indicates that there is a need to improve the immunogenicity of this vaccine. Our preliminary data indicate that MVA.HIVA stimulated proliferation of both $CD4^+$ and $CD8^+$ T cells in 75% of vaccinees. This could be an underestimate, as significant proliferation detected in a conventional thymidine incorporation assay may not be detected in a CFSE assay using the cell division index cutoff of >3, which was our criterion for a positive response in this study (12a). As $CD4^+$ T cells with proliferative and interleukin-2-secreting capacities are more abundant in patients with better virological control (15, 42), these parameters merit further scrutiny in therapeutic vaccination studies as potential correlates of vaccine-mediated control of HIV-1, and we are currently optimizing assays to detect them.

The influence of prior DNA vaccination on responses to MVA.HIVA in this study is uncertain, owing to the small number of subjects studied. Comparison of the volunteers given MVA alone and MVA 2 years after DNA vaccination showed no differences in the magnitude or breadth of either $CD8^+$ or $CD4^+$ T-cell responses detected by IFN-γ ELISPOT assay. The peak response was more likely to be attained after the first MVA vaccination in the DNA/MVA group and after the second immunization in the MVA-alone group, suggesting that DNA vaccination enhanced the efficiency of MVA vaccination. It is unlikely that a DNA vaccine would prime T-cell responses more effectively than HIV-1 itself. However, DNA vaccination has been shown to enhance the immunogenicity of a recombinant fowl pox virus/SIV vaccine in SIV-infected macaques (31). Prime-boost immunization schedules with recombinant DNA and MVA vaccines expressing the same immunogen efficiently prime CD4 T-cell responses in HIV-uninfected individuals (12a, 23). A more detailed analysis in a larger cohort is needed to assess the value of including DNA vaccines in therapeutic immunization strategies.

In conclusion, this study shows that a recombinant MVA vaccine, administered during HAART, efficiently expands both

$CD8^+$ and $CD4^+$ T cells with a favorable functional profile for containing virus replication. Supervised therapy interruptions will enable us to discern possible correlations between T-cell phenotype, function, and decay rate and viral rebound kinetics. This is essential for optimizing the immunogenicity of candidate vaccines and for elucidation of immunological correlates of virus control.

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