The Thai Phase III Trial (RV144) Vaccine Regimen Induces T Cell Responses That Preferentially Target Epitopes within the V2 Region of HIV-1 Envelope


J Immunol published online 23 April 2012
http://www.jimmunol.org/content/early/2012/04/20/jimmunol.1102756

Supplementary Material
http://www.jimmunol.org/content/suppl/2012/04/24/jimmunol.1102756.DC1

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The Thai HIV phase III prime/boost vaccine trial (RV144) using ALVAC-HIV (vCP1521) and ADSVAX B/E was, to our knowledge, the first to demonstrate acquisition efficacy. Vaccine-induced, cell-mediated immune responses were assessed. T cell epitope mapping studies using IFN-γ ELISPOT was performed on PBMCs from HIV-1–uninfected vaccine (n = 61) and placebo (n = 10) recipients using HIV-1 Env peptides. Positive responses were measured in 25 (41%) vaccinees and were predominantly CD4+ T cell-mediated. Responses were targeted within the HIV Env region, with 15 of 25 (60%) of vaccinees recognizing peptides derived from the V2 region of HIV-1 Env, which includes the α4β7 integrin binding site. Intracellular cytokine staining confirmed that Env responses predominated (19 of 30; 63% of vaccine recipients) and were mediated by polyfunctional effector memory CD4+ T cells, with the majority of responders producing both IL-2 and IFN-γ (12 of 19; 63%). HIV Env Ab titers were higher in subjects with IL-2 compared with those without IL-2–secreting HIV Env-specific effector memory T cells. Proliferation assays revealed that HIV Ag-specific T cells were CD4+, with the majority (80%) expressing CD107a. HIV-specific T cell lines obtained from vaccine recipients confirmed V2 specificity, polyfunctionality, and functional cytolytic capacity. Although the RV144 T cell responses were modest in frequency compared with humoral immune responses, the CD4+ T cell response was directed to HIV-1 Env and more particularly the V2 region.

The Journal of Immunology, 2012, 188: 000–000.

The HIV epidemic continues to grow, with 33 million persons living with HIV/AIDS and 7400 new infections daily in 2008 (1). Although recent data suggest a slowing in the rate of new infections, a HIV-1 vaccine in the setting of a comprehensive prevention program remains the most cost-effective and globally applicable public health approach to controlling the epidemic. Although no efficacy was observed for the VaxGen (injectable vaccine) (2–4), the Thai phase III prime/boost vaccine trial (RV144) demonstrated a 31% reduction in the acquisition of infection without an effect on postinfection viral load or CD4+ T cell count in infected vaccinees (5). A prior phase II trial of the identical vaccine regimen demonstrated robust humoral and cellular immune responses in vaccinees as measured by HIV-specific binding and neutralizing Ab assays and Ag-specific lymphoproliferation assays (6). Cumulative chromium release CD8+ CTL activity was detected in 27% of vaccine recipients (6) and was comparable with that reported with similar vaccine regimens using chromium release (7, 8) or IFN-γ ELISPOT (9).

Despite numerous international canarypox trials with or without an Env protein boost (10, 11), T cell epitope mapping studies after immunization with ALVAC and HIV Env protein(s) have been limited. One study using an identical vaccination schedule to
RV144 assessed cellular immune responses in HIV seronegative subjects after ALVAC (vCP205, which expresses HIV-1 subtype B gag, pro, and env) alone, in combination with a single boost of gp160 MN/LAI-2, or gp160 MN/LAI-2 alone reported stronger and broader responses to vaccine-matched HIV peptides after ALVAC prime/protein boost, compared with those of volunteers immunized with vCP205 alone (12). CD4+ T cell lines from subjects receiving the combination regimen demonstrated positive responses across the entire gp160, with the notable exception of the V3 region. More extensive T cell epitope mapping studies have been performed with HIV prime/boost vaccine strategies using DNA priming followed by either modified vaccinia virus Ankara (13) or recombinant adenovirus serotype 5 vectors (14).

Preliminary studies of RV144 cellular immune responses identified reactivity that was predominantly CD4+ T cell-mediated and directed against HIV-1 Env (5). The IFN-γ ELISPOT and intracellular cytokine staining (ICS) for IFN-γ and IL-2 assays after the stimulation of PBMCs with HIV peptide pools demonstrated a modest cell-mediated immune response. In vaccines, a greater frequency of IFN-γ ELISPOT responders was observed against Env (21 of 153; 14%) compared with Gag (9 of 148; 6%), and ICS responses were CD4+ T cell-mediated and focused on Env (47 of 142; 33%) rather than Gag (2 of 144; 1%) (5).

This study was undertaken to assess T cell responses to HIV-1 Env epitopes induced by the RV144 vaccine regimen of ALVAC-HIV (vCP1521) (Sanofi) and AIDSVAX B/E (Global Solutions for Infectious Diseases) after the completion of immunization. A comprehensive functional analysis of vaccine-induced T cell responses, including the quantification of T cell responses by IFN-γ ELISPOT at 2 wk after final vaccination, was performed. On the basis of the findings at the peak immunogenicity time point, functional characterization of T cell responses (cytokine expression, proliferation, CD107a expression, and lytic activity) was performed at 6 mo after the completion of immunization. Fig. 1 shows the time points after immunization for which cellular immune responses were assessed.

Materials and Methods

Study design

RV144 was a phase III trial conducted on the Eastern seaboard of Thailand (5). A total of 12,542 volunteers received the full injection series of vaccine or placebo. The vaccine regimen consisted of recombinant canarypox, ALVAC-HIV (vCP1521), expressing HIV circulating recombinant form (CRF) 01_AE gp120 (92TH023) with subtype B (LAI) transmembrane portion of gp41 and subtype B (LAI) gag and protease, administered at 0, 1, 3, and 6 mo. AIDSVAX B/E, a bivalent HIV gp120 vaccine containing B and CRF01_AE envelopes derived from MN and A244, respectively, was coadministered with ALVAC at 3 and 6 mo. All of the vaccines were assessed by the PBMCs had been used previously in ELISPOT studies (5). PBMCs from a subset of 600 HIV-1–uninfected volunteers who completed the RV144 injection regimen were selected for immunogenicity assessment in April 2008. A randomized sample with a 3:1 frequency of vaccine/placebo ratio was 3:1 and was aiming to test a minimum of 10 placebos for enhanced assay validity. PBMCs from an additional 50 RV144 trial participants (40 volunteers and 10 placebo recipients) collected at 24 wk after the completion of immunization were tested for polychromatic flow cytometry, lymphoproliferation, and establishment of T cell lines. PBMCs from the same subjects for the different visits could not be used for the investigations of cell-mediated responses by IFN-γ ELISPOT and ICS, because the PBMC collection in RV144 was extremely limited, 8 and 16 ml of whole blood at V8 and V9, respectively, and the subjects’ V9 PBMCs had been used previously in ELISPOT studies (5).

Cell preparation

PBMCs were prepared using 8 ml sodium citrate Vacutainer CPT according to the manufacturer’s instructions (BD Biosciences) and cryopreserved in RPMI 1640 medium (Invitrogen) containing 20% heat-inactivated fetal calf serum (Invitrogen) and 10% DMSO (Sigma-Aldrich) in a Cryo 1°C freezing container (Nalgene). Cells were stored at or less than −130°C. Immunogenicity assessments were performed on cryopreserved specimens; average cell viability was 95% (range: 80–100%) after thawing. PBMCs were thawed and rested overnight before all of the assays with the exception of ICS, where they were used immediately after thawing. To evaluate T cell type responses to the peptides in the IFN-γ ELISPOT assay, overnight-rested PBMCs were split into three aliquots at a concentration of 4 × 10^6 PBMCs per milliliter and treated with immunomagnetic Dynabeads M-450 CD4 (for CD4 depletion), Dynabeads M-450 CD8 (for CD8 depletion), or Dynabeads M-450 anti-mouse Ig (for sham depletion) (Dynal Biotech ASA). The beads and adhered cells were removed with BioMag medium, and the multiwell cell populations were washed twice in cold medium complete composed of RPMI 1400 supplemented with 4 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated normal human serum (Gemini Bioproducts) and tested as for the IFN-γ ELISPOT assay described below. The efficiency of depletion was consistently ≥95% (range: 97–99% for both CD4+ and CD8+ depletion).

Ages and peptides

The HIV CRF01_AE-derived A244 gp120 was provided by Marc Gurwith (Global Solutions for Infectious Diseases) and was identical to that contained in the AIDSVAX B/E vaccine. The 165 CRF01_AE 92TH023 Env gp160 peptides of 15-mers overlapping by 11 aa (New England Peptide) were used for the stimulation of PBMCs in the ELISPOT assays in Thailand. A separate peptide set of 138 peptides of 15–18 aa overlapping by 10–12 aa spanning CRF01_AE isolate CM235 Env protein (JPT Peptide Technologies) and a peptide pool representing immunodominant CD8+ T cell epitopes within CMV, EBV, and influenza (15). All of the peptides were used at a final concentration of 1 μg/ml.

IFN-γ ELISPOT assay

Peptides in the HIV-1 Env Ag were mapped using an IFN-γ ELISPOT assay as described previously (5). In brief, 96-well hydrophobic membrane-bottom plates (Millipore) were coated overnight at 4°C with anti-human IFN-γ mAb (final concentration of 5 μg/ml [Mabtech]). Cells (PBMCs or CD4+ or CD8+–depleted PBMCs) were resuspended in complete medium and plated at a concentration of 2 × 10^6 cells per well. In instances where T cell depletions were performed, the predetermination PBMC count per well was used to avoid CD4+ or CD8+ T cell enrichment. A panel of 26 pools of 12–14 peptides (of 165) responding to the peptides in the IFN-γ ELISPOT assay were distributed to single wells of cells in a matrix format, and the plates were incubated overnight at 37°C in 5% CO2. Wells containing cells and media only were supplemented with the equivalent concentration of DMSO as the peptide pools and served as negative controls. PHA was used as a positive control, and if there were sufficient cells, CMV pp65 and the CMV, EBV, and influenza peptide pool also were included in the assay as additional positive controls. Cells plus PHA were tested in triplicate wells. Negative controls were performed in quadruplicate. After incubation at 37°C in 5% CO2 for 20–24 h, cells were removed by washing with PBS/0.05% Tween 20 (Sigma-Aldrich). Captured IFN-γ was detected by incubation for 2 h at 37°C with biotinylated anti-human IFN-γ mAb (Mabtech) at a concentration of 2 μg/ml in PBS/0.5% BSA. After incubation, plates were washed with PBS/0.05% Tween 20, and avidin-peroxidase complex (Vectastain Elite Kit) was added for 1 h at room temperature. Unbound complex was removed by washing, and peroxidase staining was performed using 3-amino-9-ethylcarbazole substrate (Vectastain AEC Kit) according to the
CD45RO cells (CD45RA

QDot 605, anti-CD8-ethylcarbodiimide, and anti-CD3-allophycocyanin-H7. Then surface-stained with anti-CD14/CD19-PE-Cy5 (Invitrogen), anti-CD4-PBMCs for a further 5 h in the presence of anti-CD107a-PE (BD Phar

were Env peptide-pulsed overnight and washed were coincubated with the autologous EBV-transformed B lymphoblastoid cell lines (B-LCLs) that contained the CM235 Env matrix pooled peptides (24 pools containing 11–13 peptides each) or no peptides and with 2 × 10^3 irradiated autologous B-LCLs. Assays were performed in duplicate. After 2 d of incubation, the cells were pulsed with 1 µCi per well of \(^{3}H\) for 18 h, harvested using the Tomtec Mach 3M (Egg&G Wallac), and counted in a 1450 MicroBeta TriLux (Egg&G Wallac). T-cell data are shown as \(z\) scores (i.e., as reactivity in standard deviations from the median reactivity to all of the peptides). Standard deviations were determined in each experiment using this equation: \(z = (\text{median} - \text{first quartile})/0.675.\) Values above \(z\) = 3.29 were considered significant in this assay.

Cryopreserved specific T cell lines were thawed, washed, and resuspended in RPMI 1640 with 10% normal human serum. Cells were incubated with Perm/Wash (BD Biosciences) for 15 min, washed, and simultaneously surface/intracellular-stained with anti-CD45RA/CD45RO/CD4/CD8, central memory cells CD45RA/CD45RO/CCR7/CD28\(^{+}\), effector memory cells CD45RA/CD45RO/CCR7/CD25\(^{+}\), and effector cells (CD45RA/CD45RO/CCR7/CD28\(^{+}\)). A positive response was defined as ≥0.05% gated positive cells for the test Ag and the response exceeding the unstimulated control by ≥3 times.

**T cell proliferation and functional assay**

Measurement of T cell proliferation and their subsequent functional profile was performed using a modified CFSE-based lymphocyte proliferation assay (17). Cryopreserved PBMCs were thawed, washed, and resuspended in RPMI 1640 containing 10% normal human serum for immediate assay. PBMCs (1 × 10^6) were incubated for 6 h in the presence or absence of a peptide pool (1 µg/peptide/ml) representing the CM235 Env protein or the positive control staphylococcal enteroxin B (10 ng/ml). Anti-CD28-biotin and anti-CD94 mAbs (BD Pharmingen) were included at set-up, and the protein transport inhibitors monensin (GolgiStop), BD Pharmingen) and brefeldin A (Sigma-Aldrich) were added after 2 h.

Cells were washed, stained with Aqua Live/Dead (Invitrogen), washed, and resuspended in flow wash buffer (PBS with 0.5% BSA and 0.1% sodium azide), resuspended in 10 µm mouse IgG (FcR block) for 15 min, centrifuged, and surface-stained with anti-CD4/CD19-Alexa Fluor 700 (BD Pharmingen), anti-CD45RA/CD45RO/FITC (BD Pharmingen), anti-CD45RO/eFluor 650 (eBioscience), anti-CD4-QDot 605 (Invitrogen), and strep-tavidin-QDot 800 (Invitrogen) to detect the CD28-biotin mAb. Cells were fixed in 2% paraformaldehyde (Tousimis) and washed and left overnight at 4°C. On the following morning, cells were incubated with Perm/Wash (BD Biosciences) for 15 min, washed, and simultaneously surface/intracellular-stained with anti-CD45RA/CD45RO/CD28/CD4/CD8, central memory cells CD45RA/CD45RO/CCR7/CD28\(^{+}\), effector memory cells CD45RA/CD45RO/CCR7/CD25\(^{+}\), and effector cells (CD45RA/CD45RO/CCR7/CD28\(^{+}\)). A positive response was defined as ≥0.05% gated positive cells for the test Ag and the response exceeding the unstimulated control by ≥3 times.

**Generation and characterization of CD4\(^{+}\) T cell lines**

PBMCs from 10 vaccinees and two placebo recipients were cultured in complete medium. The CD4\(^{+}\) T cell lines were established as described previously (18). In brief, PBMCs at a concentration of 1 × 10^6 cells per milliliter were pulsed with CM235 Env peptide pool or CM235 Env peptide #32 (VHALFYKLDIVPIEDNK) or no peptides for 6 h at 37°C in complete medium. Cells were diluted in complete medium, plated at a concentration of 2 × 10^4 cells per milliliter in 24-well plates (Costar), and incubated at 37°C in 5% CO\(_2\). After 4 d, the cells were fed with 10 IU/ml rIL-2 (Boehringer Mannheim). After 15 d, the T cells were harvested, washed, counted, and tested in a proliferation assay to assess their specificity to CM235 Env peptides. Epitope mapping was performed using the \(^{3}H\) lymphoproliferation assay as described previously (12). The proliferative response of the expanded PBMCs was measured by incubating 3 × 10^4 T cells in a 96-well flat bottom plate (Costar) containing the CM235 Env matrix pooled peptides (24 pools containing 11–13 peptides each) or no peptides and with 2 × 10^3 irradiated autologous B-LCLs. Assays were performed in duplicate. After 2 d of incubation, the cells were pulsed with 1 µCi per well of \(^{3}H\) for 18 h, harvested using the Tomtec Mach 3M (Egg&G Wallac), and counted in a 1450 MicroBeta TriLux (Egg&G Wallac). T-cell data are shown as \(z\) scores (i.e., as reactivity in standard deviations from the median reactivity to all of the peptides). Standard deviations were determined in each experiment using this equation: \(z = (\text{median} - \text{first quartile})/0.675.\) Values above \(z\) = 3.29 were considered significant in this assay.

Frozen plasma samples collected at V9 from subjects on whom ICS and immunophenotyping were performed (30 vaccinees and 8 placebo recipients) were tested for HIV binding. Titers with the gp120 Ags used in the AIDSVAX B/E boost (MN and A244) in a standard validated ELISA as described previously (5, 19). Samples were screened for the presence of gp120 B and CRF01_AE Abs at an initial dilution of 1:50. Nonreactive
samples were assigned a reciprocal titer of 25. Reactive samples (OD > 0.200) were titrated beginning at 1:100 at 2-fold dilutions. The reciprocal end-point titer was calculated as the greater OD value of either \((2 \times \text{mean OD negative control} + 2 \times \text{SD})\) or 0.100.

**Statistical analysis**

The magnitudes of ELISPOT and ICS responses are expressed after the subtraction of the backgrounds. Data are expressed as mean or median SFCs per million PBMCs with ranges for the ELISPOT and medians with ranges for the ICS and summary data. Ab titers were expressed as geometric mean titers.

Data analyses were performed using Prism 5 (GraphPad). Comparisons of categorical data were made using Fisher’s exact test. Comparisons between continuous variables were performed using parametric (if the data followed a normal distribution) or nonparametric tests. The level of statistical significance was 5% for all of the analyses. The p values were not adjusted for multiple comparisons.

**Results**

**ELISPOT matrix mapping**

PBMCs from 153 HIV-1–uninfected vaccinated subjects were tested initially by IFN-\(\gamma\) ELISPOT to ALVAC-HIV (vCP1521) Env- and Gag-matched peptides at 6 mo after the completion of immunization (V9), of whom 14% responded to Env (median: 87 SFCs per 10^6 PBMCs; range 47–222) and 6% to Gag (median: 88 SFCs per 10^6 PBMCs; range 59–99)(5). Selecting 20 with positive ELISPOT responses to 92TH023 Env at V9, 13 of 20 (65%) subjects also were positive to Env peptides at 2 wk after vaccination (V8). An additional 41 vaccinees and 10 placebo recipients (Fig. 1) were tested with EnvVD15 and the complete 92TH023 Env peptide pool after sham, CD4+, or CD8+ T cell depletion. Five of 22 subjects were positive in the ELISPOT assay to the whole Env pool (median: 28 SFCs per 10^6 PBMCs; range: 20–44) using the cutoff described for the peptide matrix. Depletion of CD4+ T cells resulted in complete loss of ELISPOT reactivity to the Env pool (median: 0; range: 0–8 SFCs per 10^6 CD4+-depleted PBMCs), whereas CD8+ cell depletion had minimal impact on the magnitude of the ELISPOT responses, compared with whole PBMCs (median: 21; range: 0–33 SFCs per 10^6 CD8+-depleted PBMCs; \(p = 0.063\)) (Fig. 2B). Despite the earlier time point in the current study, none of the 22 subjects tested to the 92TH023 Env peptide pool to assess the T cell subset producing IFN-\(\gamma\) in the ELISPOT assay met the criteria for a positive response of \(55 \text{ SFCs per } 10^6 \text{ PBMCs} \) and four times background used in the initial immunogenicity study performed at 6 mo after the completion of immunization (5). However, this is consistent with earlier studies of this vaccine regimen, which reported that novel cellular immune responses arose up to 6 mo after the completion of immunization (6).

**Multifunctional ICS and immunophenotyping analysis**

Polychromatic flow cytometry was used to explore both functional and phenotypic profiles of the Env-specific T cells in a direct ICS
Multifunctional analysis using Boolean gating revealed that the majority of the CD4+ effector memory T cells produced more than one cytokine (58%), and as expected based upon the single-cytokine analysis, IL-2– and IFN-γ-producing cells were predominant (Fig. 3). Most of the subjects with positive responses to IL-2 also produced IFN-γ (12 of 17; 71%). Median response magnitudes within the CD4+ effector memory subset for IL-2 and IFN-γ were 0.12% (range: 0.05–0.43%) and 0.13% (range: 0.05–0.46%), respectively. A trifunctional cell subset mainly synthesized IL-2, IFN-γ, and TNF-α and represented 22% of the Ag-specific population.

**HIV-specific Ab responses**

Env-specific Ab to both strains of HIV present in AIDSVAX B/E was detected in all 30 vaccine recipients and 0 of 8 placebo recipients, with reciprocal geometric mean titers (ranges) of 1131 (100–12800) and 2016 (200–12800) to A244 and MN gp120, respectively. The titers of Env-specific Ab in the vaccine recipients were stratified on HIV-specific positive IL-2 responses in effector memory CD4+ T cells of vaccine recipients. Subjects with IL-2 responses had higher HIV-specific Ab titers compared with those of the 13 subjects without IL-2 responses to both A244 (geometric mean titer [range]: 1736 [100–12800] versus 646 [100–6400]; p = 0.015) and MN (geometric mean titer [range]: 2832 [400–12800] versus 1293 [200–12800]; p = 0.045).

**Lymphocyte proliferation functional recall assay**

Simultaneous T cell proliferation and functional profiles were measured in PBMCs from HIV-uninfected vaccine recipients using a CFSE-based flow cytometric assay after restimulation with peptide-pulsed autologous B-LCLs. This assay again showed that Env-specific CD4+ T cells were the predominant responding cells (17 of 30; 57% vaccinees). A single placebo recipient (1 of 8) showed a positive response to Env. Thirteen (43%) vaccinees demonstrated concordance with positive responses in both ICS and CFSE proliferation assays after Env stimulation. Upon restimulation after 6 d in culture, proliferating CD4+ T cells, defined as CFSElow, exhibited an unusual functional phenotype potentially capable of cytotoxicity, as measured by CD107a surface expression, accompanied by IFN-γ and TNF-α production but with minimal IL-2 production (Fig. 4). Multifunctional analysis of the proliferating T cells revealed that a large fraction (43%) of these cells did not express any of the four functions assayed. Of the functional cells, CD107a expression was detected in the majority of subsets, as expected from the single-function analysis, and was detected most commonly in combination with IFN-γ. The majority (80%) of the Ag-specific functional CD4+ T cells expressed CD107a on the cell surface, with IFN-γ being the predominant cytokine (50%), followed by TNF-α (42%), with minimal IL-2 production (19%).

**HIV-specific T cell lines**

CD4+ T cells were expanded from PBMCs of HIV-uninfected vaccinees stimulated with gp120 in culture, a procedure that
yields oligoclonal, Ag-specific T cell lines (18). These lines were tested in a lymphoproliferation assay with CM235 Env peptides in a matrix format. Expanded CD4+ T cell lines from four subjects yielded responses that were deconvoluted and assigned peptide specificities. The recognized peptides confirmed similar areas of recognition found in the IFN-γ ELISPOT assay (Table II). Epitopes within the C1 and V2 regions were recognized by T cell lines from 3 of 4 subjects and 2 of 4 subjects in the C2 and C5 regions.

**FIGURE 3.** Functional profile of vaccine-induced CD4+ T cells. PBMCs stimulated with HIV Env (CM235) peptides and the functional composition (IL-2, IFN-γ, IL-21, and TNF-α) of the Env-specific CD4+ T cells 6 mo after vaccination are shown for 19 responders. The majority (>95%) of the Ag-specific cells were of the effector memory phenotype (CD45RA−/CD45RO+/CCR7−/CD28−), and the responses are grouped and color-coded on the basis of the number of functions, corresponding to the Boolean subsets in the graph. Pie arcs show the relative amounts of each individual function. All of the possible combinations of the responses are shown as Boolean subsets in the bar graph below on the x-axis, and the median percentages of the functionally distinct cell populations are shown on the y-axis.

**FIGURE 4.** Polyfunctional analysis of proliferating HIV-1 Env-specific CD4+ T cells. The pie chart on the left shows the functional profile of all proliferating CD4+ T cells (CFSElow), whereas that on the right shows only those proliferating CD4+ T cells that are functional after restimulation with peptide-pulsed autologous B-LCLs. Pie slices correspond to the fraction of cells within the CD4+ T cell population, whereas the pie arcs show the relative amounts of each individual function. The bar graph represents Boolean subset analysis of all of the functionally distinct cells within the total CD4+ population. The x-axis shows all of the possible combinations of responses, whereas the y-axis represents the median percentage of each subset within the functionally positive CD4+ population. Results shown are generated from 17 vaccinees with positive proliferative responses by CFSE analysis.
One subject’s T cell line responded only to the C4 region of CM235 gp120. Despite using a slightly different peptide set (CM235; CRF01_AE.R5-tropic) to that used in the IFN-γ ELISPOT matrix mapping (92TH023; CRF01_AE.R5-tropic), the expanded CD4+ T cells from 2 of 4 subjects demonstrated peptide reactivity to the analogous region of 92TH023 EnvVD15, which has the identical sequence to peptide 44 and two additional C-terminal residues, asparagine and lysine (Table II).

The two expanded T cell lines (144620 and 144721) that recognized the 92TH023 EnvVD15 analog were tested in a polychromatic ICS assay after stimulation with CM235 Env pooled peptides. The cytokine production profile was predominantly multifunctional, and cells showed CD107a surface expression (Fig. 5).

The [51Cr] cytotoxicity assays were performed on 144721 and an additional four T cell lines. Table III shows the immunophenotypes of the five CD4+ T cell lines and their Env region specificities. Four of the five lines responded to peptides in the V2 region. All of the lines demonstrated cytolytic activity to the CM235 Env peptide pool (Fig. 6A), with 144721 having the greatest lytic activity despite the absence of CD8+ T cells (Table III). T cell lines 144072 and 144712 were tested further for functional cytotoxicities to the TH023 EnvVD15 analog EnvVK16. Lytic activities to EnvVK16 were observed for both lines and more strongly for 144072 (Fig. 6B). T cell line 144072 also had no measurable CD8+ T cells (Table III).

**Discussion**

This study assessed T cell responses in RV144 vaccinees by IFN-γ ELISPOT at 2 wk after immunization using the same HIV Env pool as that used in a previous study of RV144 cellular immune responses at 6 mo after the completion of immunization (5).

![Figure 5](http://www.jimmunol.org/)

*FIGURE 5.* Multifunctional profiles of Env-specific CD4+ T cell lines derived from two RV144 vaccine recipients 6 mo after vaccination. Results shown were generated after in vitro stimulation of CD4+ T cell lines expanded using gp120 A244. Each pie chart corresponds to the functional profile for each subject. Responses are grouped and color-coded on the basis of the number of functions. The pie chart summarizes the data, with each slice of the pie corresponding to the fraction of cells within the total CD4+ T cell population. Pie arcs show the relative amounts of each individual function. Bars represent the fraction of functionally distinct cells within the total CD4+ T cell population.
Table III. Immunophenotypes of T cell lines established from RV144 vaccine recipients and HIV Env recognition patterns

<table>
<thead>
<tr>
<th>Subject</th>
<th>%CD4*</th>
<th>%CD8*</th>
<th>HIV Env Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>144072</td>
<td>96</td>
<td>0</td>
<td>V2</td>
</tr>
<tr>
<td>144712</td>
<td>66</td>
<td>11</td>
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<tr>
<td>144445</td>
<td>80</td>
<td>6</td>
<td>V2</td>
</tr>
</tbody>
</table>

Cells were used as effector cells in cytotoxicity assays to the HIV CM235 Env peptide pool.

FIGURE 6. Cytotoxic function of CD4+ T cell lines measured by [51Cr] release. (A) Lytic activities of T cell lines from five vaccinated subjects to autologous B-LCLs pulsed with the HIV Env (CM235) peptide pool. (B) Lytic activities of T cell lines from two vaccinated subjects to B-LCLs pulsed with CM 235 Env V2 peptide #32 (VHALFYKLDPEDNK). Results are expressed as the percentage of specific lysis (y-axis) of the target cells at various E:T ratios (x-axis).

The mean number of Env epitopes recognized (3.6) in the RV144 vaccine group with positive responses in the IFN-γ ELISPOT assay is similar to that reported for the EuroVacc 02 phase I study (4.2) (13) and multiclade HIV-1 DNA prime/recombinant Adenovirus 5 vaccine regimen (3.3) (14) but greater than that reported for the STEP trial (1) (25).

Despite measurable CD8+ cytolytic T cells using the chromium release assay (6), direct ex vivo HIV-specific CD8+ T cell responses from RV144 subjects’ PBMCs were barely measurable (<10%) in the IFN-γ-IL-2 combination ICS assay and were equivalent to the frequency of responses seen in the placebo recipients (5). However, robust CD4+ T cell responses assessed by both 3H incorporation and the ICS assays were reported in the vaccine group (5). The finding of direct ex vivo vaccine-induced T cell immune responses being predominantly CD4+ T cell-mediated agrees with data reported from DNA prime followed by poxvirus or adenovirus boost studies (10, 11, 13, 14, 26, 27). One DNA prime/adenovirus serotype 5 boost HIV-1 vaccine trial reported a greater frequency (93%) of HIV-specific CD4+ T cell responses compared with CD8+ T cell responses (71%) 4 wk after recombinant adenovirus 5 boosting (14).

Polychromatic flow cytometry of PBMCs after stimulation with a heterologous CRF01 AE Env peptide set to that used in the IFN-γ ELISPOT assay again demonstrated that the vaccine elicited direct ex vivo T cell responses that were exclusively CD4+ T cell-mediated and also multifunctional. Multifunctional (defined as the production of IL-2 in addition to effector function, such as the production of IFN-γ) T cells (28) are thought to be important for the control of a number of virus infections (28–31). The combination of IL-2 and IFN-γ production capability is consistent with the phenotypic effector memory classification of these cells (28).

The finding that up to 6 mo after the completion of immunization the HIV-specific CD4+ T cells were of the effector memory phenotype, with most cells producing IL-2, would allow effector function, proliferation, and induction of CD8+ T cell antiviral activity (14, 30, 32). Effector memory CD4+ T cells producing IL-2 are thought to be important in the development of IgG-secreting plasma B cells and have been reported to be positively associated with Ab titers to hepatitis B surface Ag up to 9 years after immunization (33), a vaccine that like the RV144 regimen induces robust humoral immune responses with limited direct ex vivo T cell responses. The finding that the vaccine group with IL-2–producing effector memory T cells had higher gp120 Ab titers compared with the group without would support this observation.

To our knowledge, this is the first use of polychromatic flow cytometry in a clinical trial using this vaccine regimen and demonstrates that the ALVAC + gp120 B/E vaccine regimen induced multifunctional and potentially cytolytic CD4+ T cell responses up to 6 mo after the completion of immunization. All of the CD4+ T cells induced by the vaccine were of the effector memory phenotype, which is similar to that reported previously for a DNA/NYVAC clinical vaccine trial (13).
One limitation of our study was that we did not assess the phenotype of the responding cells at 2 wk after the completion of immunization, so the profile may differ from that at the 24-wk time point, but the EuroVacc 02 study did not detect a difference in the phenotype of vaccine-induced CD4+ T cells with increased time from the completion of immunization (13). The presence of vaccine-induced CD4+ T cell responses has been reported to correlate with virus control in a vaccine-challenge study using ALVAC gag-pol-env prime/gp120 boost vaccine regimen and simian-HIV challenge (34). The lymphocyte proliferation functional recall assay showed that the cytokine profile of functional CD4+ T cells implied a terminal effector function (31, 35).

The establishment of polyfunctional HIV-1 Env-specific CD4+ T cell lines that also express CD107a and are capable of cytotoxic activity further verified the findings of the lymphocyte proliferation functional recall assay, although it should be stressed that these findings are not directly ex vivo. However, one pilot study with HIV-infected volunteers compared the delayed type hypersensitivity skin test assay with autologous CD4+ T cell line lymphoproliferation using identical HIV Env V3 peptides and reported excellent concordance, suggesting that the lines may be measuring a relevant in vivo immune function (36), although prolonged culture may change the phenotype associated with a particular TCR genotype.

HIV-1–specific cytotoxic CD4+ T lymphocytes using a variety of assays have been described in HIV-1 infection (37–42). A study of acute HIV infection in the United States (presumably HIV subtype B) using CD4+ T cell clones derived from a volunteer subject at 16 d postinfection just before antiretroviral therapy initiation and was not observed again despite repeated testing (up to 683 d postinfection), despite the infecting virus not showing sequence variation within this region (42). Other studies of cellular immune responses in HIV-infected cohorts have inferred that this V2 epitope may be recognized but have not identified it as a highly recognized region (43–45).

Appay et al. (38) speculated that the generation of CD4+ CTLs could be to control viruses targeting MHC class II-expressing cells and/or downregulate MHC class I expression. In natural infection, these perforin-containing CD4+ T cells possessed a terminally differentiated phenotype (CD45RO+/CCR7+/CD28–), with the production of TNF-α and IFN-γ, but not IL-2 (38), similar to that of the cytotoxic CD4+ T cell lines established after HIV-1 Env restimulation in this study. CD4+ CTLs also have been described after immunization with the HIV gp160 protein subunit, although cloned CD4+ T cell lines were used (46). It has been proposed that the generation of cytotoxic CD4+ T cells in addition to CD8+ CTLs by HIV vaccine regimens would provide the advantage of dual MHC class I and II recognition and that the greater diversity of MHC class II epitope recognition would allow a greater breadth of virally infected targets (35, 47). Cytolytic CD4+ T cells also may be important in the control of several herpesviruses, measles viruses, and dengue viruses (48).

Although the direct ex vivo cellular immune response induced by the RV144 vaccine regimen was modest in relation to the humoral immune response as measured by binding Ab, the observation that the RV144 vaccinees recognized T cell epitopes predominantly within the V2 loop of HIV-1 Env is intriguing given the location of a known ligand (LDI) for the α4β7 integrin within this region (20). Substitution of the aspartic acid in the LDI region with hydrophilic basic amino acids has been found to abrogate virus infectivity, whereas substitution with negatively charged amino acids maintained virus infectivity with heightened neutralization sensitivity (49).

The lack of published data from epitope mapping studies on the predominance of EnvVD15 T cell epitope responses in HIV-infected subjects to date (43, 44, 50, 51) suggests that this epitope may be masked on the virion surface or not easily produced and/or presented in MHC class II or that cells with this specificity are infected preferentially with the virus and destroyed during infection (42, 52).

The RV144 vaccine regimen induced modest ex vivo T cell responses with respect to frequency and magnitude as measured by IFN-γ ELISPOT and ICS compared with those of other HIV vaccine regimens with either poxvirus (13, 53) or adenovirus (54, 55) vector boosting after DNA priming. However, humoral immune responses and Ag-specific lymphoproliferative responses measured by HIV binding Ab to gp120 and 3H, respectively, were robust (5). A similar vaccine regimen combination of ALVAC prime and HIV Env boost has shown some protection in animal models. An infant macaque model of ALVAC-SIV expressing gag-pol-env administered at 0, 1, and 3 wk followed by multiple low-dose oral challenges of SIV at 4 wk also demonstrated partial protection from infection, despite minimal HIV-1–specific IFN-γ ELISPOT responses in immunized animals (56). Another ALVAC-HIV-1 gag-pol-env-nef prime/HIV-1 Env protein boost study followed by mucosally administered simian-HIV as a challenge in macaques reported only modest (33%) HIV-specific CTL activity, but there was a reduction in infection in the immunized animals compared with that in the unimmunized group, in addition to protection from CD4+ T cell loss after virus challenge (34).

Characterizing the immune response in studies such as this can help to generate potential correlates for formal testing. Despite the modest T cell responses induced by the RV144 trial regimen, the finding of preferential cellular immune responses to gp120 V2 epitopes led to studies on Ab responses to gp120 V2 induced by the vaccine regimen in HIV-uninfected vaccinees and was found in 92% of subjects at 2 wk after the completion of immunization (N. Karasavvas, unpublished data). A case-control study using multiple assays for cellular and humoral immune responses in RV144 vaccinees who became HIV-infected compared with those who remained uninfected reported an inverse correlation between Ab levels to a conformational V1/V2 epitope and HIV infection but no other potentially protective immune responses (57).

The comprehensive functional capacity of the Env-specific CD4+ T cells induced by the RV144 vaccine regimen needs to be assessed further. Ongoing work, including additional HIV vaccine trials using poxvirus prime/HIV Env protein boost, is seeking to elucidate correlate(s) of protection induced by the RV144 vaccine regimen by allowing larger specimen collection volumes for more detailed immunogenicity studies (58).

Acknowledgments

We thank the RV144 study participants for allowing this study to occur, the Thai Ministry of Public Health staff in Rayong and Chon Buri for invaluable assistance on this trial, and the Vaccine Clinical Trial and Data Management staff at Mahidol University. We also thank Josephine H. Cox, Nicos Karasavva, and Peter Gilbert for helpful discussions and review of the manuscript, Kanyasiri Kongnonk and Boot Keawboon for work on the Supachai ELISPOT assays, and Bonnie Slike for assistance with the tables. Gratitude also is expressed to Viseth Ngauy for continued support of this project.
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Disclosures
The authors have no conflicting financial interests.

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