

Induction of Multifunctional Human Immunodeficiency Virus Type 1 (HIV-1)-Specific T Cells Capable of Proliferation in Healthy Subjects by Using a Prime-Boost Regimen of DNA- and Modified Vaccinia Virus Ankara-Vectored Vaccines Expressing HIV-1 Gag Coupled to CD8⁺ T-Cell Epitopes†

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A double-blind randomized phase I trial was conducted in human immunodeficiency virus type 1 (HIV-1)-negative subjects receiving vaccines vectored by plasmid DNA and modified vaccinia virus Ankara (MVA) expressing HIV-1 p24/p17 gag linked to a string of CD8⁺ T-cell epitopes. The trial had two groups. One group received either two doses of MVA.HIVA (2× MVA.HIVA) (*n* = 8) or two doses of placebo (2× placebo) (*n* = 4). The second group received 2× pThr.HIVA followed by one dose of MVA.HIVA (*n* = 8) or 3× placebo (*n* = 4). In the pThr.HIVA-MVA.HIVA group, HIV-1-specific T-cell responses peaked 1 week after MVA.HIVA vaccination in both ex vivo gamma interferon (IFN-γ) ELISPOT (group mean, 210 spot-forming cells/10⁶ cells) and proliferation (group mean stimulation index, 37), with assays detecting positive responses in four out of eight and five out of eight subjects, respectively. No HIV-1-specific T-cell responses were detected in either assay in the 2× MVA.HIVA group or subjects receiving placebo. Using a highly sensitive and reproducible cultured IFN-γ ELISPOT assay, positive responses mainly mediated by CD4⁺ T cells were detected in eight out of eight vaccinees in the pThr.HIVA-MVA.HIVA group and four out of eight vaccinees in the 2× MVA.HIVA group. Importantly, no false-positive responses were detected in the eight subjects receiving placebo. Of the 12 responders, 11 developed responses to previously identified immunodominant CD4⁺ T-cell epitopes, with 6 volunteers having responses to more than one epitope. Five out of 12 responders also developed CD8⁺ T-cell responses to the epitope string. Induced T cells produced a variety of anti-viral cytokines, including tumor necrosis factor alpha and macrophage inflammatory protein 1β. These data demonstrate that prime-boost vaccination with recombinant DNA and MVA vectors can induce multifunctional HIV-1-specific T cells in the majority of vaccinees.

Over 95% of people infected with human immunodeficiency virus type 1 (HIV-1) live in the developing world, where both social and economic factors preclude effective prevention programs and limit access to antiretroviral therapy (70). The production of a prophylactic HIV-1 vaccine, even if partially efficacious (3), is a priority to effectively curb infection rates and HIV-1-related deaths in these regions.

No studies have so far evinced an immune correlate of protection, whether antibody or T-cell mediated, from either HIV-1 infection or progression to disease. Antibodies produced in response to HIV-1 infection afford little if any control

of viremia, appearing to select for virus escape mutants (60, 75). HIV-1 antibody vaccines so far developed have used inactivated virus or recombinant envelope proteins that are capable of effective neutralization of laboratory HIV-1 strains (reviewed in reference 40). However, these vaccines have either been largely ineffective against primary HIV-1 isolates or, if neutralizing (48), require the induction of high titers difficult to achieve through vaccination (31, 44, 64, 69).

In contrast, T cells, particularly CD8⁺ T cells, exert some control over HIV-1 viremia and progression to disease in natural infection. In the acute stage of infection, decreased HIV-1 viremia is associated with the detection and expansion of virus-specific CD8⁺ T cells prior to the detection of neutralizing antibodies (10, 38, 54). In chronic HIV-1 infection, about 5% of patients infected do not progress to AIDS. Resistance to disease in these patients and resistance to infection in highly exposed seronegative patients is associated with the detection of HIV-1-specific CD8⁺ T cells and some major histocompat-

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ibility complex class I alleles (15, 35, 47, 63). These observations in human HIV-1 infection remain correlative, but direct evidence of a role for T cells has been demonstrated in non-human primate resistance to simian immunodeficiency virus. Depletion of CD8⁺ T cells prior to challenge increased susceptibility of macaques to infection with simian immunodeficiency virus (34). Subsequent studies showed that the induction of high frequencies of T cells in macaques following vaccination with various T-cell vaccines lowered viremia in animals challenged with the highly aggressive SHIV.89.6P strain (2, 7, 61, 66). Despite the achievement of vaccine-induced protection in nonhuman primates, in one animal protection was lost when a mutation occurred in a single immunodominant CD8⁺ T-cell epitope, demonstrating that following infection CD8⁺ T cells exert pressure on viral selection (5). Examples of CD8⁺ T-cell viral escape mutants and, more recently, emergence of an escape mutant prior to viral rebound has also been described in human HIV-1 infection (55, 57).

We have developed recombinant DNA(pThr.) and modified vaccinia virus Ankara (MVA) vaccines expressing a common immunogen, HIVA, which consists of consensus HIV-1 clade A gag protein linked to a string of immunodominant CD8⁺ T-cell epitopes (27). Immunogenicity studies using these vectors in rodents and macaques elicited high frequencies of CD8⁺ T cells after vaccination (27, 74). In our previous phase I trial, this vaccine regimen appeared poorly immunogenic, showing detectable T-cell responses in only 10% of vaccinees (A. Guimaraes-Walker, unpublished data). We re-evaluated this study and designed a new double-blinded trial. In this study, higher doses of both DNA and MVA were given to volunteers to better reflect doses that are immunogenic in smaller animals. The bleed schedule was also changed to incorporate a bleed at the peak of the vaccine-induced T-cell response, which occurs 1 week after the MVA boost (49, 71). Ex vivo gamma interferon (IFN- γ) ELISPOT is widely used in HIV-1 clinical trials to measure the frequency of effector T cells induced by vaccination; however, the contributions of IFN- γ or the relative roles of effector versus central memory T cells to host immunity to HIV-1 remain unknown. We sought to better characterize the T-cell response induced following vaccination by performing a range of assays to detect functions, including T-cell proliferation and cytokine production. We demonstrated that the pThr.HIVA-MVA.HIVA regimen elicited specific, multifunctional T cells in the majority of vaccinees.

MATERIALS AND METHODS

Vaccines. pThr.HIVA and MVA.HIVA (26) were produced in accordance with Good Manufacturing Practice by COBRA Therapeutics (Keele, United Kingdom) and IDT (Roßblau, Germany), respectively. Both vectors express the HIV-1 clade A consensus sequence gag p24 and p17 coupled to a string of partially overlapping CD8⁺ T-cell epitopes, which are derived from the gag, pol, nef, and env proteins and are restricted by 17 different HLA class I alleles.

Vaccination schedule. The trial consisted of two groups (Table 1). The first ($n = 12$) received two doses of 4 mg of pThr.HIVA ($n = 8$) (designated 2 \times pThr.HIVA) or saline placebo ($n = 4$) given intramuscularly 4 weeks apart. Four weeks later, subjects receiving pThr.HIVA were immunized with 10⁸ PFU of MVA.HIVA intradermally. Subjects receiving placebo received a third placebo vaccination (3 \times placebo) ($n = 4$). All subjects received their first vaccination. However, due to a trial delay, only 3 of the 12 subjects in the second group completed the original schedule. Of the remaining nine, three did not complete

TABLE 1. Trial schedule

Group	Vaccine	Wk	Dose	Route	No. of subjects in group (placebos)
pThr.HIVA-MVA.HIVA	pThr.HIVA	0	4 mg	i.m. ^a	12 (4)
	pThr.HIVA	4	4 mg	i.m.	
	MVA.HIVA	8	10 ⁸	i.d. ^b	
2 \times MVA.HIVA	MVA.HIVA	0	10 ⁸	i.d.	12 (4)
	MVA.HIVA ^c	4	10 ⁸	i.d.	

^a i.m., intramuscular in upper arm.

^b i.d., intradermal in upper arm.

^c Due to a trial delay, while all subjects in this group received their first vaccination, only 3 out of 12 received the second vaccination on schedule. Of the remaining nine, six received their second vaccination 6 to 9 months after the first vaccination, and three did not complete the schedule.

the schedule and six received their second vaccination after a delay of 6 to 9 months.

Blood separation. Peripheral blood mononucleocyte (PBMC) separation was performed within 2 h of blood receipt. Blood was layered onto Ficoll (Sigma-Aldrich, St Louis, MO) and centrifuged (40 min, 400 \times g, without brake) at room temperature. Following centrifugation, the cellular interface was removed, diluted in Hanks buffer (Sigma-Aldrich), and then recentrifuged. Cells were washed once more with 50 ml RPMI (Sigma-Aldrich) and then suspended in 10 ml RPMI for counting. Cells were counted using a Coulter Z1 Counter (Beckman-Coulter, Buckinghamshire, United Kingdom). Trypan blue exclusion (Sigma-Aldrich) was used to enumerate the percentage of viable cells.

Antigens. Peptide pools 1 to 4 consist of 22 to 23 15-mer peptides, overlapping by 11 amino acids and spanning the gag component of the HIVA immunogen (Anaspec, San Jose, CA) described previously (52, 53). Pool 90 is a combined pool of pools 1 to 4 consisting of 90 peptides. Pool 10 is identical to pool 2 but lacks peptide 42. In IFN- γ ex vivo and cultured ELISPOT assays, the T-cell response to pool 90 stimulation correlated significantly with responses to its components, pools 1 to 4 and 10 ($P < 0.0001$). Pool 9 contains the epitopes in the CD8⁺ T-cell string. The FEC pool contains influenza virus, Epstein-Barr virus, and cytomegalovirus CD8⁺ T-cell epitopes (20). In all assays, peptides were used at the following concentrations: pool 90 and FEC at 1.5 μ g/ml; pools 1 to 4, individual peptides, and pool 9 at 2 μ g/ml. Negative or "mock" controls contained 0.45% dimethyl sulfoxide in culture media. Staphylococcal enterotoxin B (SEB) (Sigma S4881) and phytohemagglutinin (PHA) (Sigma SL4144) were positive controls and were used at 10 μ g/ml.

Ex vivo IFN- γ ELISPOT. The use of peptide plates prepared prior to the start of the trial improved the quality of the data produced by minimizing operator error and improving replicates. Peptides were made to a double concentration in R-10 (10% fetal bovine serum F4135, 86% RPMI 1640, 2 mM L-glutamine G7513, 1 \times penicillin-streptomycin solution P7539, 10 mM HEPES buffer H0887, 1 mM sodium pyruvate solution S8636; Sigma-Aldrich, Dorset, United Kingdom). Peptides were aliquoted into 96-well round-bottom plates in volumes of 65, 120, or 175 μ l/well, sealed with TopSeal A/100 plate sealers (Perkin Elmer, Boston, Mass.), and then frozen at -80°C until use. On the day of the ELISPOT assays, seals were removed and peptide plates were thawed at 37 $^{\circ}\text{C}$. PBMC (4×10^6 cells/ml R-10) were aliquoted using a multichannel pipette in 50- μ l volumes into a 96-well MAIPS4510 plate (Millipore Stonehouse, Gloucestershire, United Kingdom) previously coated with mouse anti-human IFN- γ MAb-1-D1K (Mabtech, Nacka Strand, Sweden). Peptides from the peptide plate were mixed and then added in 50- μ l volumes to the ELISPOT plate containing PBMC. Plates were incubated at 37 $^{\circ}\text{C}$, 5% CO₂ for 18 to 20 h. Following incubation, PBMC were discarded, and plates were washed six times with PBS/0.05% Tween 20. One-hundred microliters of secondary MAb-7-B6-1 (Mabtech Nacka, Strand, Sweden) used at 1 μ g/ml of 0.5% bovine serum albumin-phosphate-buffered saline (PBS) was added, and plates were incubated at room temperature for 2 to 4 h. Plates were washed, and 100 μ l/well of peroxidase avidin-biotin complex PK6100 was added (Vector Labs, Peterborough, United Kingdom). Finally, plates were washed three times with PBS/0.05% Tween 20 and then three times with PBS and developed with AEC substrate solution (AEC tablets; Sigma A6926 diluted in acetate buffer) for 4 min. The reaction was stopped with water washes. In the interleukin-7 (IL-7)-IL-15-supplemented ex vivo IFN- γ ELISPOT assay, cells were supplemented with 5 ng/ml of each cytokine (R&D Systems,

TABLE 2. Prevacination statistics and positive response definition for each assay performed in the trial

Assay antigen stimulant	Unit	Assay statistics				Definition of a positive response				
		Prevacination or placebo response <i>n</i>	Mean ^a	Median ^a	99%	Highest prevacination level ^a	Highest placebo level ^a	Positive response ^a	Replicate variation	Requirements for controls
Ex vivo IFN-γ ELISPOT for pool 90, pools 1 to 4, and pool 9	SFC/10 ⁶ cells	252	0.49	0.00	8.75	10.0	5.0	1 pool = 38 ^b , 2 pools = 30 ^b	CV ^c < 70%	Mock < 55, >4× mock
IL-7-IL-15 ex vivo IFN-γ ELISPOT and for pool 90, pools 1 to 4, and pool 9	SFC/10 ⁶ cells	220	3.79	0.00	92.5	81.3	163.8	1 pool > 163; 2 pools > 93	CV < 70%	>4× mock
Cultured IFN-γ ELISPOT performed on pool 9 STCL	SFC/10 ⁶ cells	54	-6.0	-6.3	68.8	56	69			
Cultured IFN-γ ELISPOT performed on pool 90 STCL	SFC/10 ⁶ cells	281	31	12.5	263	269	269			
Cultured IFN-γ ELISPOT for total of pools 90 and 9	SFC/10 ⁶ cells	335	25	6.3	263	269	269	1 pool = 300 ^d	CV < 70%	Mock < 250, >4× mock, phyhemagglutinin > 1,000
[³ H]thymidine proliferation for pools 90 and 9	SI	233	0.94	0.86	3.18	3.58	3.05	5		SEB > 0.5, >4× mock
CFSE proliferation for pools 90 and 9	PI	161	-0.002	0.000	0.015	0.015	0.010	0.03		Mock < 0.03, ^e >4× mock
WB IFN-γ ICS for pool 90, pools 1 to 4, and pool 9	% CD3/CD4 CD69/IFN-γ ⁺	292	0.001	0.000	0.025	0.022	0.054	1 pool = 0.055		

^a Data are for mock (background)-subtracted response of cells stimulated with peptide antigens for pool 90, pools 1 to 4, and pool 9.

^b This cutoff is based on IAVI CORE laboratory data from trial 006 (*n* = 1,216 assays, highest prevacination or placebo response, 38 SFC/10⁶ cells).

^c CV, coefficient of variation.

^d Due to detection of an HIV-A-specific response to pool 10 detectable in prevacination samples and healthy laboratory workers, a positive response in this assay required >300 SFC/10⁶ in wells stimulated with pool 9 or a similar response to pool 90 together with at least one from pool 1, 3, or 4 or peptide 42. Assays with positive responses to pool 90 and pool 10 (pool 10 is identical to pool 2 but lacks peptide 42) only were excluded from analysis.

^e Minimum of events in the CD3⁺ CD4⁺ gate required was dependent on the following background levels: 0.00 to 16,000 events, 0.01 to 20,000 events, 0.02 to 30,000 events, and 0.03 to 35,000 events.

Oxon, United Kingdom), and the assay was performed as described above. MAIP plates were read using an AID machine and AID ELISPOT 3.1.1 HR software (Autoimmun Diagnostika, GmbH Strassberg, Germany), calibrated weekly using an AID Master 33 plate.

[³H]thymidine proliferation assays. To minimize contamination, antigen stocks were frozen at twice the final concentration in 1.0- to 2.0-ml aliquots prior to the start of the trial. To perform the assay, 10⁵ PBMC/100 μl RAB-10 (R-10 replacing fetal calf serum with pooled human AB serum Sigma H1513) in quadruplicate wells was incubated with equal volumes of mock, pool 90, pool 9, or SEB for 120 ± 4 h at 37°C, 5% CO₂. Each well containing PBMC was then pulsed with 1 μCi/20 μl of [³H]thymidine TRA120 ([³H]thymidine) (Amersham Biosciences, Buckinghamshire, United Kingdom) and left at 37°C, 5% CO₂ for an additional 16 to 20 h. [³H]thymidine incorporation was measured using a Topcount Microplate Scintillation Counter (Perkin-Elmer), and results were expressed with the following formula: stimulation index (SI) = geometric mean of antigen-stimulated cells/geometric mean of mock-stimulated cells.

CFSE proliferation assays. PBMC (1 × 10⁶/ml) were labeled with 0.8 μM carboxy-fluorescein diacetate succinimidyl ester (CFSE; Invitrogen, Paisley, United Kingdom) for 10 min in the dark and then washed three times with PBS. Cells were then cultured in RAB-10 at 37°C, 5% CO₂ for 6 days with mock, pool 90, pool 9, or SEB. Cells were stained with CD3 allophycocyanin (BD 345767), CD4 PerCPcy5.5 (BD 341654), and CD8 phycoerythrin (BD 345773), and at least 5,000 events were acquired using a FACScalibur flow cytometer (BD 34012422). Analysis was performed using MODFIT^{LT} Software. Results are expressed with the following formula: proliferation index (PI) = (sum of the cells in all generations)/(computed number of original parent cells theoretically present at the start of the experiment). The PI is proportional to the percentage of cells that have proliferated in culture and is also weighted for the number of divisions that cells have undergone.

IFN-γ whole-blood intracellular cytokine staining (WB-ICS). Nine-hundred microliters of blood/tube was incubated in a water bath with 100 μl of a 10× concentration antigen for 1 h at 37°C. Blood was stimulated with mock, pool 90, pools 1 to 4, pool 9, and SEB. Following stimulation, 10 μg/ml of brefeldin A (Sigma B7651) was added, and tubes were incubated in a water bath for 5 h at 37°C. Following incubation, 20 mM EDTA was added, and blood was incubated in the dark for 10 min. Erythrocytes were then lysed using FACSLyse (BD 349202), and the suspension was frozen at -80°C. To stain, vials were thawed quickly at 37°C, permeabilized (BD 340973) with FACSPerm (BD 340973), and then stained using CD3 allophycocyanin (BD 345767) and antibodies from the Fastimmune CD4 Intracellular kit (BD 340970). Cells were fixed with Cellfix (BD 340181) and then acquired using a FACScalibur flow cytometer (BD 34012422).

Cultured IFN-γ ELISPOT assay. Three short-term cell lines (STCL) were produced for each volunteer and then stimulated in IFN-γ ELISPOT using prealiquoted peptide plates. Specifically, cells (2 × 10⁶/ml) were stimulated with pool 90, pool 9, or FEC. Cells were cultured at 37°C, 5% CO₂ for 11 to 13 days in RAB-10, receiving a saturating concentration of IL-2 (1,800 U/ml) at days 3 and 7. One milliliter of RAB-10/well was added at day 7, plus additional RAB-10 at days 8 to 10 as needed. Early on day 10, cells were washed three times with sterile PBS and then left in fresh RAB-10 for 25 to 35 h at 37°C, 5% CO₂. This step is key to decreasing background in subsequent assays (data not shown). On day 11, cells (4 × 10⁴/well for pool 90 and pool 9 STCL, 2 × 10⁴/well for FEC STCL) were set up in IFN-γ ELISPOT as described above. Residual cells were fed with IL-2 and then washed on day 13, and either the assay was repeated or peptide mapping was performed on day 14.

The greater sensitivity of the cultured IFN-γ ELISPOT assay identified a CD4⁺ T-cell response to pool 90 and its component pool 2 (peptides 23 to 44) of the gag region of HIVA. This T-cell response was identified in around 40% of prevacination bleeds and healthy laboratory workers. Preliminary studies using blood from healthy laboratory workers suggested that this response may be due to peptide 42 within pool 2. For this study, pool 2 was then split into peptide 42 and pool 10 (pool 2 lacking peptide 42), and where necessary, cultured IFN-γ ELISPOT assays were repeated, testing peptide 42 and pool 10 separately. However, T-cell responses detected in cultured IFN-γ ELISPOT assays in prevacination and placebo assays were specific for pool 10 and not peptide 42. On this basis, all positive T-cell responses to pool 90 that were mapped as pool 10 were assumed not to be vaccine induced and were excluded from analysis prior to unblinding (Table 2).

Depletions and peptide mapping. A major advantage of the cultured IFN-γ ELISPOT assay is that more detailed studies such as depletions and peptide mapping could be obtained with low background counts (<12 spot-forming cells (SFU)/well), low variation across replicate wells (<70% coefficient of variation), and by using very few cells (4 × 10⁴ cells/well). Cells from STCL were routinely

TABLE 3. Summary of all peak T-cell responses^a to pool 90 and positive responses to pool 9 (in parentheses) induced by vaccination

Vaccination group (n = 8) ^k	Subject no.	Assay and T-cell response					
		Cultured IFN- γ ELISPOT	[³ H]thymidine proliferation	Ex vivo IFN- γ ELISPOT	IL-7-IL-15 IFN- γ ELISPOT	CFSE proliferation	IFN- γ WB ICS
pThr.HIVA-MVA.HIVA	1	7,769^b (5,925)	224.2	959 (279)	2165 (718)	0.56	0.46 (0.18)^c
	2	10,431^d (475)	11.6	103	166 ^e	0.07	0.01
	3	3,206^b	44.4	514	ND ^f	ND	0.02
	4	4,181^b	7.33	36^g	86	0.03	0.01
	5	1,463^b	8.5	31	128	0.01	0.03
	6	388^b	4.28	30	96	0.00	0.02
	7	413^b	0.93	4	18	0.01	0.01
	8	1,769^b	1.59	5	13	0.01	0.01
MVA.HIVA	9	2,494 (906)	0.36	28	114^h (165)	ND	0.01
	10	(1,250)	0.15	-1	-3	ND	ND
	11	(356)	0.97	0	15	ND	0.00
	12	3,838	0.55	6	ND	ND	0.00
	13	280 ⁱ	1.01	0	16	ND	0.00
	14	0	0.3	3	-18	ND	0.00
	15	0 ^j	0.69	-1	31	ND	0.00
	16	-25	0.17	11	16	ND	0.00

^a Background-subtracted responses are shown. Unless otherwise indicated, responses shown are from week 9 in the pThr.HIVA-MVA.HIVA group and week 1 in the 2 \times MVA.HIVA group. Positive T-cell responses as defined in Table 2 are in boldface.

^b Positive T-cell response from week 10 (2 weeks post-MVA.HIVA). No assays were performed at week 9.

^c Peak pool 9-specific T-cell response detected at week 10.

^d Positive T-cell response from week 12 (4 weeks post-MVA.HIVA). There were insufficient cells to perform the assay at week 10.

^e A value of 116 SFC/10⁶ cells does not equal a positive response for this subject, because 116 SFC is less than 4 \times mock at this time point (see Table 2).

^f ND, no data were available for pool 90-specific T-cell responses at week 9 or week 1. No positive responses were recorded at any other time points.

^g A value of 6 SFC/10⁶ cells is a positive response, because this subject had a response of >30 SFC/10⁶ cells to two peptide pools (see Table 2).

^h A value of 114 SFC/10⁶ cells is a positive response, because this subject had a response of >93 SFC/10⁶ cells to two peptide pools (see Table 2).

ⁱ Pool 1 response is shown. The greater proportion of the pool 90 response was attributable to pool 10 and therefore was excluded from the definition of a vaccine-induced T-cell response (see Table 2).

^j Prevacination data were available for pool 9 STCL only.

^k No positive responses were recorded for the placebo group (subjects 17 to 24).

CD8 T cell depleted (and in some cases, also CD4 depleted) using Miltenyi technology prior to the resting step (Miltenyi Biotec, Gladbeck, Germany). Briefly, cells were chilled on ice, washed, and incubated with Miltenyi beads (CD8, 40 μ l; CD4, 30 μ l) for 15 to 20 min at 4°C. Cells were again washed and run through a Miltenyi MS column according to the manufacturer's directions. This method typically resulted in >95% depletion of cell subsets (data not shown). For peptide mapping, when a positive T-cell response to a peptide pool was observed, cells (4 \times 10⁴ cells/well) were then tested in duplicate against the individual peptides (2 μ g/ml) contained in that peptide pool using the cultured IFN- γ ELISPOT assay. Individual peptide-specific T-cell responses were then confirmed in quadruplicate at the subsequent bleeds, again using cultured IFN- γ ELISPOT assays.

Luminex assays. The pool 90 and pool 9 STCL assayed in cultured IFN- γ ELISPOT were also used in multicytokine Luminex assays. As with the [³H]thymidine proliferation assays, peptides were frozen in aliquots at a double concentration prior to the start of the trial and then thawed as needed. Cells (10⁵/100 μ l in duplicate) were stimulated for 12 h in 96-well round-bottom plates with equal volumes of either mock, pool 90, pool 9, or SEB. Supernatants were frozen at -80°C, and cytokine concentration was measured with a Bio-plex human cytokine 17-plex panel (per manufacturer's instructions) and measured using a Luminex array reader (Bio-Rad Laboratories, Hertfordshire, United Kingdom).

Statistical analysis. All criteria for defining a positive response for each assay were defined prior to unblinding and are summarized in Table 2. Statistical analyses were performed by the EMMES Corporation using SAS software. Nonparametric tests of inference were applied due to the small numbers of observations and the nonnormal distribution of response measures. Three-way comparisons across groups were performed using the Kruskal-Wallis analysis of variance (ANOVA) test. Two-way comparisons used the Wilcoxon, two-tailed signed rank test. Intra- and interassay correlations were estimated by Spearman's nonparametric correlation coefficient. For correlations between assays, the mean response per volunteer over the 1-, 2-, and 4-week visits after the last MVA.HIVA vaccination was used. Results are defined as statistically significant at the 5% level, with statistical significance of the correlation between IFN- γ and the other 16 cytokines from the Luminex assay adjusted for multiple comparisons. Unless otherwise stated, graphs show the arithmetic mean of pool 90- or pool

9-specific T-cell responses in each treatment group. Supplemental figures available online graph individual data points for each subject against group mean.

RESULTS

Vaccination with pThr.HIVA-MVA.HIVA induced HIV-1-specific T-cell responses in ex vivo assays. No positive, HIV-1-specific T-cell responses (defined in Table 2) were detected in ex vivo IFN- γ ELISPOT at any time point in the schedule in the 2 \times MVA.HIVA group or in subjects receiving placebo (Table 3). In the pThr.HIVA-MVA.HIVA group, subjects received 4 mg pThr.HIVA at weeks 0 and 4, but no HIV-1-specific T-cell responses were detected until 1 week after the MVA.HIVA boosting vaccination, when positive responses to the gag region of HIVA were detected in four out of eight subjects (Table 3, Fig. 1A, and Fig. S1A in the supplemental material). The mean gag-specific response peaked 1 week after the MVA.HIVA vaccination (group mean, 209.8 SFC/10⁶ cells; range, 3.8 to 957.2 SFC/10⁶ cells) and then declined 2 and 4 weeks postvaccination (Fig. 1A and Fig. S1A in the supplemental material). Group comparisons 1 and 2 weeks after the last MVA.HIVA vaccination showed that the frequency of gag-specific T cells was significantly greater in the pThr.HIVA-MVA.HIVA group than in both the MVA.HIVA (week 1, $P = 0.0023$; week 2, $P = 0.0153$) and placebo (week 1, $P = 0.0017$; week 2, $P = 0.0175$) groups.

On the same assay plate as the standard ex vivo IFN- γ ELISPOT, cells were stimulated with HIVA peptides in culture media supplemented by IL-7-IL-15. These cytokines have

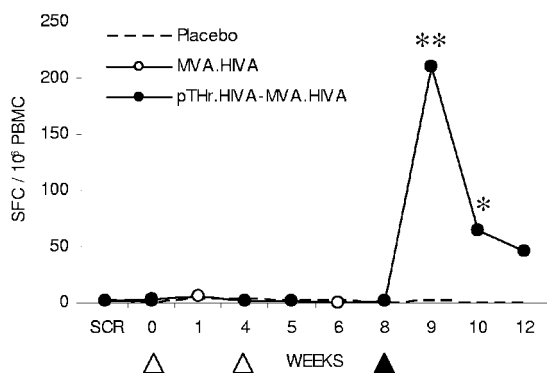
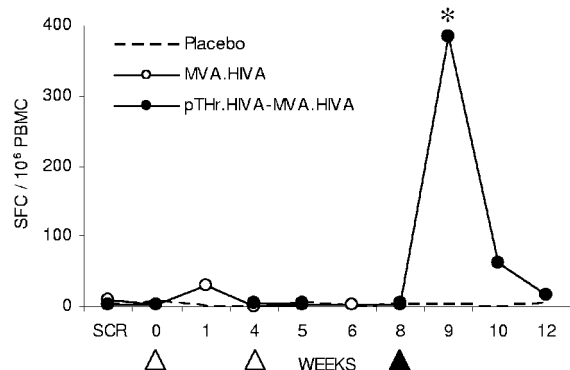
A. *Ex vivo* IFN- γ ELISPOTB. IL-7/IL-15-supplemented *ex vivo* IFN- γ ELISPOT

FIG. 1. MVA.HIVA boosts pThr.HIVA-induced T-cell responses measured in *ex vivo* IFN- γ ELISPOT. Subjects (eight per group) were vaccinated with pThr.HIVA-MVA.HIVA (black circles), MVA.HIVA alone (open circle), or saline placebo (dashed line). Mean background-subtracted responses are shown for *ex vivo* IFN- γ ELISPOT (A) and IL-7-IL-15-supplemented *ex vivo* IFN- γ ELISPOT (B). Arrows indicate vaccinations in the pThr.HIVA-MVA.HIVA group of pThr.HIVA (open arrow) and MVA.HIVA (closed arrow). *, $P < 0.05$; **, $P < 0.01$ by ANOVA (Kruskal-Wallis test). SCR, bleed taken at screening visit.

been shown to rescue cells from apoptotic death (13), and from preliminary experiments we confirmed a previous report that the addition of IL-7-IL-15 increased the frequency of response the *ex vivo* IFN- γ ELISPOT (33 and data not shown). As was expected, the peak response was higher in the IL-7-IL-15-supplemented than the standard *ex vivo* IFN- γ ELISPOT assay (compare Fig. 1A and B, as well as Fig. S1A and S1B in the supplemental material). In addition, the kinetics of response observed in the *ex vivo* IFN- γ ELISPOT was comparable in the IL-7-IL-15-supplemented IFN- γ ELISPOT (Fig. 1B and Fig. S1B in the supplemental material), and a very strong correlation ($R = 1$, $P < 0.0001$) was observed between assays (Table 4). The IL-7-IL-15-supplemented IFN- γ ELISPOT assay identified a vaccine-induced T-cell response specific for gag and the CD8⁺ T-cell string of HIVA in subject 9 (defined in Table 2) that was not detected by the standard *ex vivo* IFN- γ ELISPOT

(Table 3). However, overall fewer positive responses were recorded following vaccination in IL-7-IL-15-supplemented ELISPOT than the standard *ex vivo* IFN- γ ELISPOT (Table 3) because backgrounds were more variable in this assay, forcing the cutoff for a positive response to be raised to four times that of the standard *ex vivo* IFN- γ ELISPOT (Table 2).

Vaccination with DNA.HIVA-MVA.HIVA induced HIV-1-specific T cells capable of proliferation. T-cell-proliferative responses were measured by [³H]thymidine incorporation. Positive proliferation (SI > 5) specific to the gag region of HIVA was detected in five out of eight subjects receiving pThr.HIVA-MVA.HIVA (Table 3). No proliferation was observed in either the 2 \times MVA.HIVA group or subjects receiving placebo (Table 3). The kinetics of the proliferative response in the pThr.HIVA-MVA.HIVA group was similar to that observed in the *ex vivo* IFN- γ ELISPOT, peaking 1 week after the MVA.HIVA boost and then decreasing fourfold 2 weeks after vaccination (Fig. 2A and Fig. S2 in the supplemental material). Statistical analysis confirmed that the peak proliferation in the pThr.HIVA-MVA.HIVA group was significantly greater than that in the MVA.HIVA-only and placebo groups ($P = 0.0265$). Again, highly significant correlations were observed between [³H]thymidine incorporation and *ex vivo* IFN- γ ELISPOT pool 90 responses (these responses were CD4⁺ cell mediated; see below) (Table 4).

Using the CFSE flow-based proliferation assays, no HIV-1-specific T-cell proliferation (defined in Table 2) was observed in either the placebo or 2 \times MVA.HIVA group (Table 3). In two subjects in the pThr.HIVA-MVA.HIVA group also responding in [³H]thymidine proliferation assays, gated CD3⁺ CD4⁺ cells produced a positive PI in response to pool 90 stimulation 1 week after the MVA.HIVA vaccination (Table 3, CFSE staining for subject 1, which is shown in Fig. 2B). Overall, there was no significant difference between groups at either 1 or 2 weeks after the MVA.HIVA vaccination (week 1, $P = 0.087$; week 2, $P = 0.292$). There were insufficient cells to perform the CFSE assay at week 9 on subject 3, who produced the second strongest [³H]thymidine proliferation (SI > 40) and second highest peak *ex vivo* IFN- γ ELISPOT response in the trial (514 SFC/10⁶ PBMC). It is likely that this volunteer would also have produced a positive PI as measured in the CFSE proliferation assay at that visit. No HIV-1-specific T-cell proliferation was observed for any vaccinee in the CD3⁺ CD8⁺ T cells (data not shown).

IFN- γ WB-ICS is less sensitive than the *ex vivo* IFN- γ ELISPOT assay. The flow cytometry-based WB-ICS assay is increasingly used as an alternative to *ex vivo* ELISPOT assays, because it allows additional phenotyping of antigen-specific T cells. Only one of eight vaccinees in the pThr.HIVA-MVA.HIVA group produced a positive response (defined in Table 2) in the IFN- γ WB-ICS assay (Table 3 and Fig. 3A). This subject developed detectable CD8⁺ T-cell responses to the CD8⁺ T-cell epitope string and CD4⁺ T-cell responses to pool 90 (Table 3 and Fig. 3B) and pools 1 to 4 (data not shown) 1 week after the MVA.HIVA boost. Even so, the percentage of IFN- γ -producing cells in the CD3⁺ CD4⁺ T-cell population in the pThr.HIVA-MVA.HIVA group was significantly higher than that in both the 2 \times MVA.HIVA and placebo groups at both 1 ($P = 0.0144$) and 2 ($P = 0.0223$) weeks after the last MVA.HIVA vaccination (Fig. 3A and Fig. S3 in the supple-

TABLE 4. Correlations^a (upper line) and *P* values^b (lower line) between assays calculated from pool 90 background-subtracted responses in subjects receiving vaccines^c

Assay	Ex vivo IFN- γ ELISPOT	IL-7-IL-15 IFN- γ ELISPOT	IFN- γ WB-ICS	CFSE proliferation	Cultured IFN- γ ELISPOT	[³ H]thymidine proliferation
Ex vivo IFN- γ ELISPOT	1	1.00 <0.001	0.881 0.004	0.512 0.194	0.571 0.139	0.929 0.001
IL-7-IL-15 IFN- γ ELISPOT		1	0.88109 0.004	0.512 0.194	0.571 0.139	0.929 0.001
IFN- γ WB-ICS			1	0.537 0.170	0.476 0.233	0.905 0.002
CFSE proliferation				1	0.854 0.007	0.634 0.091
Cultured IFN- γ ELISPOT					1	0.714 0.047
[³ H]thymidine proliferation						1

^a Spearman correlation coefficients. *n* = 8. Spearman nonparametric rank order correlation (eight observations per assay).

^b *P* < 0.01 is considered significant.

^c Significant correlations are in boldface.

mental material). These significant differences between groups were maintained when subject 1 (Fig. 3B), the one positive responder in the pTHr.HIVA-MVA.HIVA group, was removed from the analysis (data not shown). The absolute percentages at the peak of response are small (group mean for pTHr.HIVA-MVA.HIVA, 0.065%; placebo, 0.003%; 2 \times MVA.HIVA, 0.002%) and were arguably not biologically relevant. However, they significantly correlated with both the ex vivo IFN- γ ELISPOT and [³H]thymidine assays (Table 4), suggesting that the IFN- γ WB-ICS assay may be sufficiently sensitive to discriminate between vaccine regimens.

Cultured IFN- γ ELISPOT was the most sensitive T-cell assay. By expanding the HIV-1-specific T-cell response during culture, the cultured IFN- γ ELISPOT assay was able to detect T-cell responses that were not detected using any of the above assays. Vaccination with pTHr.HIVA induced positive T-cell responses to pool 90 in one subject (subject 2) at 4 and 8 weeks (3,493 and 2,300 SFC/10⁶ cells, respectively; see Fig. S4A in the supplemental material). Following MVA.HIVA vaccination, eight out of eight vaccinees receiving pTHr.HIVA-MVA.HIVA and four out of eight subjects in the 2 \times MVA.HIVA group produced positive HIV-1-specific T-cell responses (Table 3). The mean response 2 weeks after MVA.HIVA boosting in the pTHr.HIVA-MVA.HIVA group was 24-fold greater than that at the corresponding time point in the ex vivo IFN- γ ELISPOT and demonstrated the magnitude of T-cell expansion achieved by cell culture (compare Fig. 1 and Fig. 4). Cultured IFN- γ ELISPOT detected CD8⁺ T-cell responses specific to the CD8⁺ T-cell epitope string in 5 out of 16 vaccinees (Tables 3 and 5 and Fig. S4B in the supplemental material). T-cell responses in three out of five of these vaccinees were mapped to the A*0301-restricted peptide TVYYGVPVWK from the env protein (Table 5). No positive T-cell responses were recorded in the placebo treatment group, since all pool 90 responses detected in the placebo controls (dashed line in Fig. 4A) were mapped to pool 10 (data not shown) and, for reasons explained in Materials and Methods, were excluded from analysis. Cultured IFN- γ ELISPOT T-cell responses

correlated with the CFSE proliferation assay and to some extent with the [³H]thymidine incorporation assay (Table 4). This would be expected, considering that the higher frequencies of response detected by cultured IFN- γ ELISPOT required proliferation of HIV-1-specific T cells during the culture period. All responses detected in ex vivo IFN- γ ELISPOT or IFN- γ WB-ICS assays were also detected in cultured IFN- γ ELISPOT assays (Table 3). The lack of correlation between the cultured and ex vivo IFN- γ ELISPOT assays (Table 4) reflects the increased sensitivity of the cultured IFN- γ ELISPOT that identified vaccine-induced T-cell responses not detected by the ex vivo assays (Table 3).

Vaccine-induced HIV-1-specific T cells secrete multiple cytokines. The cytokine milieu secreted by short-term cell lines produced for analysis by cultured IFN- γ ELISPOT was measured using Luminex technology. The group means of the cytokine concentrations in the culture supernatants for vaccinees in the pTHr.HIVA-MVA.HIVA group are shown for the pool 90 stimulation (Fig. 5A). IFN- γ production in cultured supernatants significantly correlated with the frequency of pool 90-specific T-cell responses in cultured IFN- γ ELISPOT (*P* = 0.0001). Production of IFN- γ in culture supernatants in the pool 90 STCL (Fig. 5A) also correlated significantly with the secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor alpha (TNF- α), IL-1 β , MIP-1 β , IL-4, IL-6, IL-10, and IL-13 (*P* < 0.003). Only two vaccinees (subjects 1 and 2) in this treatment group produced pool 9-specific T-cell responses in cultured IFN- γ ELISPOT assays (Table 3). Figure 5B shows the individual cytokine profile of the CD8⁺ T-cell-mediated pool 9 response for subject 1, whose stimulated PBMC produced high levels of both IFN- γ and MIP-1 β .

Vaccine-induced HIV-1-specific T-cell responses were mostly mediated by CD4⁺ T cells. The more sensitive cultured IFN- γ ELISPOT assay detected positive T-cell responses in all subjects in the pTHr.HIVA-MVA.HIVA group. The subjects responded to one to eight different epitopes (data not shown), and the range of the overall response was broad (390 to 1,000

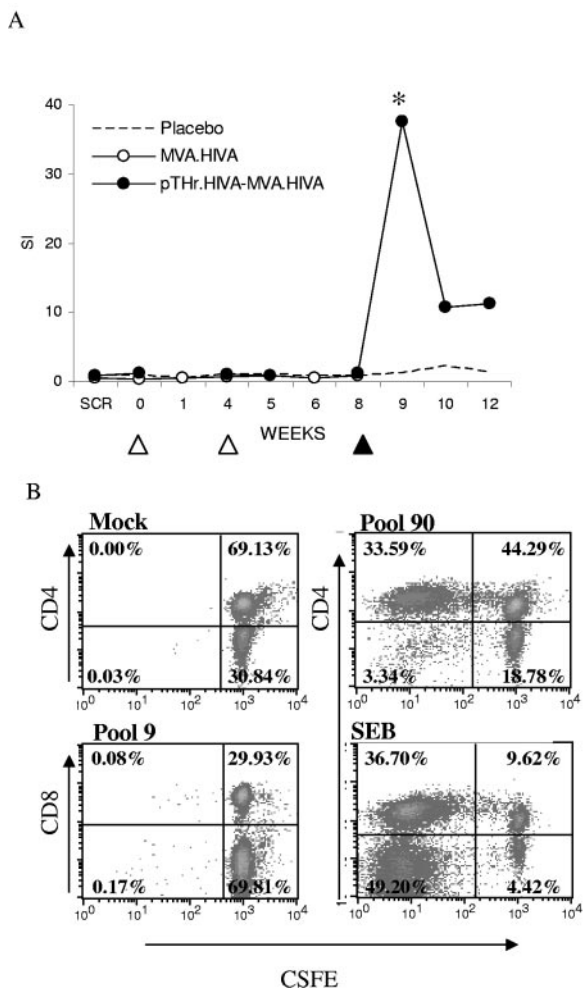


FIG. 2. MVA.HIVA boosts pTHr.HIVA-induced HIVA-specific proliferative responses. Subjects (eight per group) were vaccinated with either pTHr.HIVA-MVA (black circles), MVA.HIVA alone (open circle), or saline placebo (dashed line). Mean SI per group are shown in the ^3H thymidine proliferation assays (A). A positive proliferation index (PI) as measured by CFSE staining was observed in subject 1 from the pTHr.HIVA-MVA group 1 week after receiving MVA.HIVA (B). Arrows indicate vaccinations in the pTHr.HIVA-MVA group of pTHr.HIVA (open arrow) and MVA.HIVA (closed arrow). *, $P < 0.05$ by ANOVA (Kruskal-Wallis test). SCR, bleed taken at screening visit.

SFC/ 10^6 PBMC) (Table 3). The magnitude of the T-cell response in nondepleted versus CD8^+ T-cell-depleted cell cultures stimulated with pool 90 and tested in cultured IFN- γ ELISPOT assays was unchanged (data not shown) in all responding vaccinees, indicating that vaccination with pTHr.HIVA-MVA.HIVA induced T-cell responses that were mostly mediated by CD4^+ T cells (summarized in Table 5). T-cell responses were detected to 29 out of 90 peptides (data not shown) in pool 90, with four out of eight vaccinees in the pTHr.HIVA-MVA.HIVA group responding to two or more epitopes (Table 5). The more common CD4 epitopes detected following vaccination of HIV-1-negative subjects with pTHr.HIVA-MVA.HIVA corresponded to previously defined immunodominant CD4 T-cell epitopes capable of promiscuous

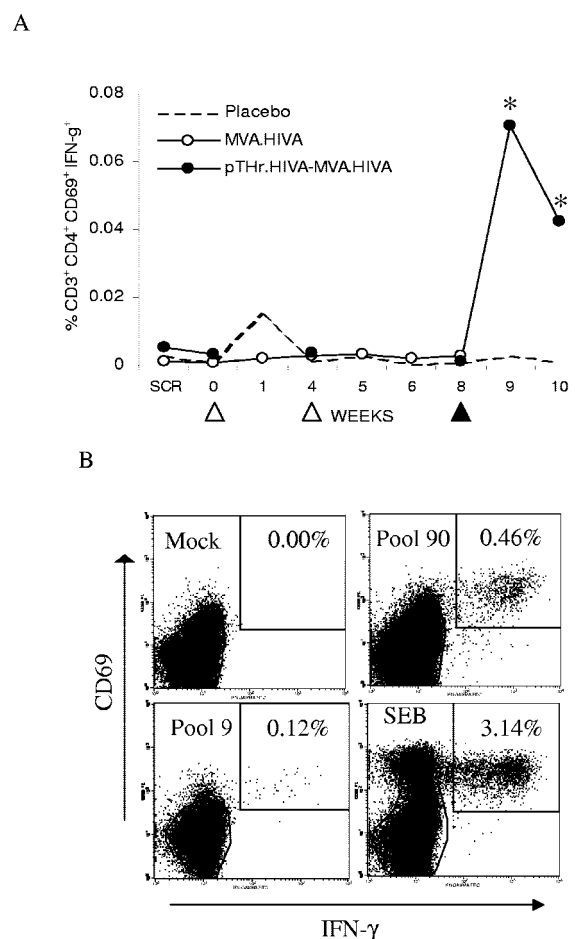


FIG. 3. MVA.HIVA boosts pTHr.HIVA-induced T-cell responses measured in WB-ICS assays. Subjects (eight per group) were vaccinated with pTHr.HIVA-MVA.HIVA (black circles), MVA.HIVA alone (open circle), or saline placebo (dashed line). (A) The mean percentages of background-subtracted events in the $\text{CD3}^+ \text{CD4}^+ \text{CD69}^+ \text{IFN-}\gamma^+$ gate are shown for IFN- γ WB-ICS. (B) Despite significant differences in response following vaccination between groups, only subject 1 from the pTHr.HIVA-MVA.HIVA group produced a positive response 1 week after the MVA.HIVA boost (Table 2). Arrows indicate vaccinations in the pTHr.HIVA-MVA group of pTHr.HIVA (open arrow) and MVA.HIVA (closed arrow). *, $P < 0.05$; **, $P < 0.01$ by ANOVA (Kruskal-Wallis test). SCR, bleed taken at screening visit.

binding to multiple HLA-DR alleles identified in HIV-1-infected patients and are listed in Table 5 (36).

DISCUSSION

Studies comparing the phenotype and function of T cells in patients with either progressive or nonprogressive HIV-1 disease have described differences in CD4 and CD8 T-cell proliferation (58, 62), production of cytokines (28), expression of memory and activation markers (16), and production of cytolytic (4) and noncytolytic antiviral factors (reviewed in reference 21). Together these reports suggest that a protective T-cell response to HIV-1 infection may involve different subpopulations of multifunctional T cells (56). The implication for

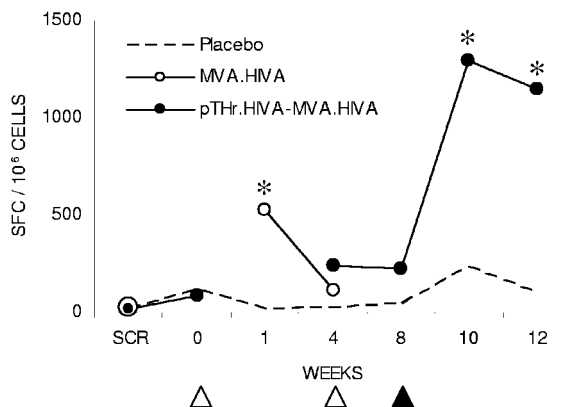
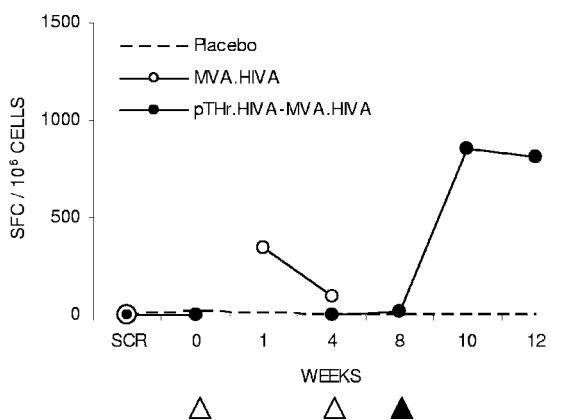
A. Cultured IFN- γ ELISPOT: Pool 90 STCLB. Cultured IFN- γ ELISPOT: Pool 9 STCL

FIG. 4. MVA.HIVA boosts pThr.HIVA-induced T-cell responses measured in cultured IFN- γ ELISPOT. Subjects (eight per group) were vaccinated with pThr.HIVA-MVA.HIVA (black circles), MVA.HIVA alone (open circle), or saline placebo (dashed line). Mean background-subtracted responses per group are shown for pool 90 STCL (A) and pool 9 STCL (B). Arrows indicate vaccinations in the pThr.HIVA-MVA.HIVA group of pThr.HIVA (open arrow) and MVA.HIVA (closed arrow). *, $P < 0.05$ by ANOVA (Kruskal-Wallis test). SCR, bleed taken at screening visit.

HIV-1 vaccine development is that with no identified correlate of immune protection, multiple measures of T-cell function must be made. Few trials testing prophylactic HIV-1 T-cell vaccines have been published. Vaccines so far tested have included recombinant DNA, MVA, canary pox, and lipopeptides (11, 14, 23, 41, 51, 52). Each study used different T-cell assays and applied different criteria to define the vaccine-induced T-cell response. This, combined with the small numbers within each treatment group, makes it difficult to compare the immunogenicity of the regimens and establish the kinetics of the T-cell response induced following vaccination.

In this study, we approached these problems by measuring multiple functions of T cells induced by vaccination and ap-

plying stringent criteria (determined from prevaccination and placebo data to define positive responders [Table 2]). We demonstrated that four out of eight and eight out of eight subjects that received MVA.HIVA alone and pThr.HIVA prime-MVA.HIVA boost vaccination regimens, respectively, had detectable HIV-1-specific T-cell responses in their circulating PBMC. These T cells were mainly CD4⁺, their frequencies peaked at 1 week after the first MVA.HIVA vaccination, and they readily proliferated and produced multiple cytokines to specific antigenic stimuli. Together, these data showed that the pThr.HIVA vaccine primed in vivo HIV-1-specific T cells and that the MVA.HIVA vaccine was able to further expand these cells in a majority of vaccinees.

Several assays measuring T-cell functions were used throughout the study. The results obtained in ex vivo IFN- γ ELISPOT, IL-7-IL-15-supplemented ex vivo IFN- γ ELISPOT, IFN- γ WB-ICS, and proliferation assays correlated significantly (Table 4). In particular, all four assays indicated the same kinetics of the vaccine-induced T-cell responses, which peaked 1 week after receiving the first MVA.HIVA dose and decreased by 50% by the following week. In our hands, the IFN- γ WB-ICS assay was less sensitive than the ex vivo IFN- γ ELISPOT assay, whereas the most sensitive assay was the cultured IFN- γ ELISPOT assay. The cultured IFN- γ ELISPOT assay detected HIV-1-specific T-cell responses in all vaccinees in the pThr.HIVA-MVA.HIVA group and in four out of eight vaccinees receiving MVA.HIVA alone. The in vitro peptide-stimulated culture in this assay expanded both CD4⁺ and CD8⁺ T-cell responses, which were often undetectable in ex vivo assays, and showed more than 24-fold increases in the frequency of specific T cells measured by ex vivo IFN- γ ELISPOT. The kinetics of the T-cell responses observed are consistent with those induced in response to viral infections in which the effector T-cell population decays rapidly, leaving a small subset of long-lived memory T cells. We are currently using multiparameter flow-based assays to examine the effector/memory phenotype of the T cells induced in this vaccine study.

The relatively few cells needed for the cultured IFN- γ ELISPOT assay allowed peptide mapping and depletion studies to be performed on all subjects. Consistent with other published clinical vaccine studies, the DNA/MVA vaccination regimen employed in this study induced predominately CD4⁺ T-cell responses to the expressed protein (49, 71). The induction of multifunctional and strongly proliferative CD4⁺ T cells is important for the maintenance of functional CD8⁺ T cells (68). Here, 11 out of 12 responders produced CD4⁺ T-cell responses, of which 6 responded to between two and eight peptides. Interestingly, CD4⁺ T-cell responses in healthy vaccinees corresponded to previously identified CD4 immunodominant T-cell responses, which suggests HIV-1 infection does not subvert major histocompatibility complex class II processing of the gag protein. Together these results show that the gag protein which is immunodominant in HIV-1 infection (18) is also highly immunogenic in healthy volunteers, substantiating its inclusion in HIV-1 vaccines.

CD8⁺ T-cell responses were detected in 5 out of 16 vaccinees and were all specific to the CD8⁺ T-epitope string. These data further support the previously reported observation (67) that CD8⁺ T-cell epitopes separated by short linker sequences and not in the context of the native protein can be processed and presented in vivo. Disappointingly, no responses to the

TABLE 5. CD4⁺ (with more than two subjects responding) and CD8⁺ HIV-1-specific T-cell responses (three out of five subjects mapped) induced in healthy subjects by vaccination

HIVA peptide	Position of immunogen ^a	Protein	Sequence	CD4 dependent	No. of vaccinees responding	HLA
6	22–36	p24	AWVKVIEEKAFSPEV ^b	+	2	U ^c
13	49–64	p24	PODLNMMMLNIVGGHQA	+	2	U
35	138–152	p24	LNKIVRMYSPVSILD ^b	+	2	U
42	166–180	p24	DRFFKTLRAEQATQE ^b	+	3	U
61	242–256	p17	GGKLDaweiKIRLRPG	+	2	U
68	270–284	p17	SRELERFALNPSLLE ^b	+	4	U
69	274–289	p17	ERFALNPSLLETAE ^b	+	4	U
76	302–316	p17	TSEELKSLFVTVATL ^b	+	2	U
77	306–320	p17	LKSLFNTVATLYCVH ^b	+	2	U
102	430–440	gp120	TVYYGVPVWK	–	3	A*0301

^a Described in reference 26.

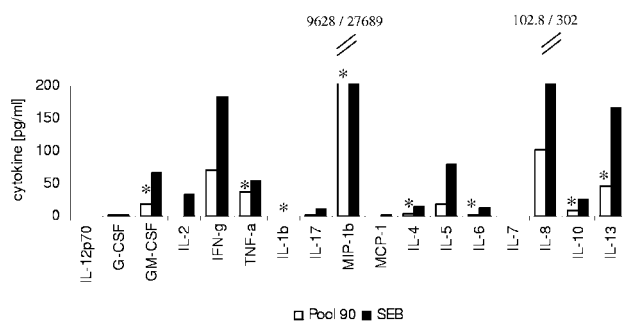
^b Peptide contains previously described promiscuously binding, immunodominant CD4 T-cell epitopes in HIV-1-infected patients (36).

^c U, HLA restriction of peptide-specific response in vaccinee not defined in this study.

many CD8⁺ T-cell epitopes previously described in the gag region of the vaccine (1) were detected in the pTHr.HIVA-MVA.HIVA group. Both this study and published data on pTHr.HIVA-MVA.HIVA vaccination show weaker immuno-

genicity following pTHr.HIVA vaccination and an overall poorer induction of CTL responses (both breadth and magnitude) than vaccination with the same vectors in animal models (30, 43, 73). A likely reason for these results is the lower doses per body surface area used in humans compared to those used in experimental animals. The induction of T-cell responses following DNA vaccination appears to be dose dependent, with stronger immunogenicity in humans being reported following vaccination with higher doses of DNA (22). Given that higher doses of DNA may not be practical or cost-effective for a developing-world disease like HIV-1, alternative strategies to increase the immunogenicity of DNA vaccines may involve using different routes of delivery (8) or delivering DNA vaccines with adjuvants or cytokines, which have been shown to increase the frequency of vaccine-induced CD8⁺ T-cell responses and improve protection from simian-human immunodeficiency virus challenge in macaques (reviewed in references 6 and 12). Such DNA vaccine formulations are now in or are entering phase I trials.

A. Luminex ELISA: Pool 90 STCL



B. Subject 1: Luminex ELISA Pool 9 STCL

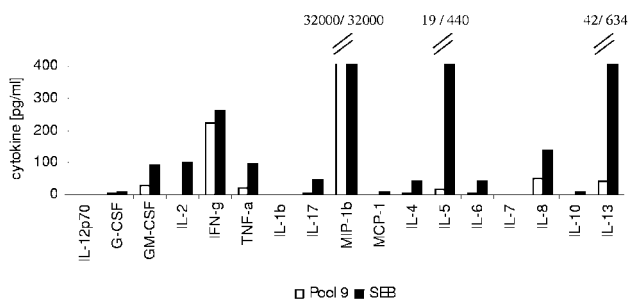


FIG. 5. HIVA-specific cells expanded in culture secrete multiple cytokines. Culture supernatants from pool 90 and pool 9 STCL derived from week 10 bleeds after stimulation with mock, pool 90 or pool 9, or SEB. The average mock-subtracted cytokine concentrations in supernatants from the pool 90 STCL for subjects in the pTHr.HIVA-MVA.HIVA group (*n* = 7) are shown in panel A, and cytokine concentrations in supernatants from the pool 9 STCL for subject 1 from the pTHr.HIVA-MVA group are shown in panel B. *, *P* < 0.05 (Spearman's nonparametric correlation coefficient corrected for multiple comparisons).

Data obtained in this study following MVA-vectored vaccine delivery in humans (45, 46) are more consistent with results obtained from animal experiments that demonstrate that recombinant MVA is capable of boosting low-frequency T-cell responses (25, 65). Despite the lack of detectable T-cell responses in ex vivo assays following pTHr.HIVA vaccination, a single MVA.HIVA vaccination boosted T-cell responses in the majority of subjects that were detected in ex vivo IFN- γ ELISPOT, proliferation, and cultured IFN- γ ELISPOT assays. Studies in HIV-1 chronic infection have shown that preexisting HIV-1-specific CD4⁺ and CD8⁺ T-cell responses could be boosted by recombinant MVA vaccination (19, 21a). Together these data suggest that recombinant MVA is capable of boosting both CD4⁺ and CD8⁺ HIV-1-specific T cells, but improved vaccine modalities capable of efficient T-cell priming are needed.

In chronic HIV-1 infection, while the presence of HIV-1-specific CD4 and CD8 T cells is associated with long-term nonprogression, neither the breadth nor the magnitude of the HIV-1-specific effector T cells correlated with viral load (1, 9). In this study, vaccine-induced T-cell responses were mostly undetectable by 4 weeks after the MVA.HIVA boost (week

12). Extending the culture period to 10 days with IL-2 addition showed HIV-1-specific T-cell responses were still capable of strong proliferation at week 12. These cell cultures displayed a TH1 cytokine profile producing significantly higher levels of IFN- γ and TNF- α than IL-4 and IL-10 than did mock-stimulated cells. Supernatants from HIV-1-specific CD4 T-cell cultures showed a significant association between the production of IFN- γ and several other cytokines, including TNF- α , MIP-1 β , and GM-CSF, previously shown to have antiviral functions (17, 29, 42, 72, 76).

These data suggest that the cultured IFN- γ ELISPOT assay may expand cell populations different from or additional to that detected by ex vivo assays. Two recent studies in malaria have shown that T-cell responses detected by IFN- γ -cultured IFN- γ ELISPOT protocols but not ex vivo IFN- γ ELISPOT assays correlated with protection from malaria; first, against natural infection in field trials in The Gambia, and second, against controlled malaria challenge in healthy subjects receiving prime-boost vaccination regimens with recombinant DNA or viral vectors (37, 59). In the study by Keating et al. (37), the authors show that strong cultured IFN- γ ELISPOT responses were detectable 6 months after vaccination with nonpersistent vaccine vectors and suggest that the cultured IFN- γ ELISPOT is expanding long-lived or memory T cells (37). Studies are now assessing the longevity and phenotype of the T-cell response induced by pThr.HIVA-MVA.HIVA vaccination in this study using cultured ELISPOT, tetramer, and multiparameter flow-based assays.

The question of whether memory or effector T cells mediate protection from human disease is debated in the literature. Adoptive transfer and gene deletion studies in mice have shown that memory T cells can mediate protection from viral infection in the absence of antigen (32, 39, 50, 77). Whether memory cells or antigen-dependent effector T cells mediate protection against HIV-1 infection is unknown. Recombinant adenovirus expressing HIV-1 antigens induces a more long-lived effector T-cell response with a higher proportion of specific CD8 T cells than MVA vaccination (66), possibly due to the different proportion of CD4/CD8 T cells induced, the biodistribution and persistence following infection, and/or the higher doses of adenovirus used in vaccination. The level of cell-mediated immunogenicity induced following adenovirus vaccination can be inhibited by preexisting adenovirus seropositivity. We suggest that the strong boosting ability of MVA demonstrated in this and other studies could successfully increase adenovirus-induced effector T-cell responses (24) in both adeno-seropositive and adeno-seronegative patients and may be a promising prime-boost regimen for future HIV-1 vaccine studies.

In conclusion, the results of this study demonstrate that small-scale, placebo-controlled, detailed clinical trials can successfully and rapidly demonstrate the immunogenicity of vaccine regimens. We show that a heterologous prime-boost regimen using DNA- and MVA-vectored vaccines can prime multifunctional HIV-1-specific T cells capable of rapid proliferation in eight out of eight vaccine recipients; however, further development of this vaccine strategy is needed to induce more durable and higher frequencies of HIV-1-specific CD8 T cells. Parallel studies in primates are investigating the role of low-frequency T-cell populations expandable by culture in pro-

tection from challenge. Future clinical studies will focus on determining the longevity of the HIV-1-specific T-cell responses induced by DNA and MVA vaccination, developing novel delivery mechanisms and methods of adjuvanting of DNA vaccines, and partnering recombinant MVA with newly emerging and promising HIV-1 vaccine candidates.

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