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T Cell Immunogenicity, Gene Expression Profile, and Safety of Four Heterologous Prime-Boost Combinations of HIV Vaccine Candidates in Healthy Volunteers: Results of the Randomized Multi-Arm Phase I/II ANRS VRI01 Trial

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Heterologous prime-boost strategies are of interest for HIV vaccine development. The order of prime-boost components could be important for the induction of T cell responses. In this phase I/II multi-arm trial, three vaccine candidates were used as prime or boost: modified vaccinia Ankara (MVA) HIV-B (coding for Gag, Pol, Nef); HIV LIPO-5 (five lipopeptides from Gag, Pol, Nef); DNA GTU-MultiHIV B (coding for Rev, Nef, Tat, Gag, Env gp160 clade B). Healthy human volunteers (n = 92) were randomized to four groups: 1) MVA at weeks 0/8 + LIPO-5 at weeks 20/28 (M/L); 2) LIPO-5 at weeks 0/8 + MVA at weeks 20/28 (L/M); 3) DNA at weeks 0/4/12 + LIPO-5 at weeks 20/28 (G/L); 4) DNA at weeks 0/4/12 + MVA at weeks 20/28 (G/M). The frequency of IFN-γ–ELISPOT responders at week 30 was 33, 43, 0, and 74%, respectively. Only MVA-receiving groups were further analyzed (n = 62). Frequency of HIV-specific cytokine-positive (IFN-γ, IL-2, or TNF-α) CD4$^+$ T cells increased significantly from week 0 to week 30 (median change of 0.06, 0.11, and 0.10% for M/L, L/M, and G/M, respectively), mainly after MVA vaccinations, and was sustained until week 52. HIV-specific CD8$^+$ T cell responses increased significantly at week 30 in M/L and G/M (median change of 0.02 and 0.05%). Significant whole-blood gene expression changes were observed 2 wk after the first MVA injection, regardless of its use as prime or boost. An MVA gene signature was identified, including 86 genes mainly related to cell cycle pathways. Three prime-boost strategies led to CD4$^+$ and CD8$^+$ T cell responses and to a whole-blood gene expression signature primarily due to their MVA HIV-B component. *The Journal of Immunology*, **2022, 208: 2663–2674.**

Since the mid-1980s, several HIV vaccine candidates have been brought into clinical development. Major efforts have been deployed to establish vaccine strategies capable of eliciting robust protective and/or antiviral T and B cell immune responses and to narrow down the best-in-class combinations of both prophylactic and therapeutic vaccines (1–3). Nevertheless, to date, no efficacious HIV vaccines are available.

Various therapeutic vaccine strategies have been tested in phase I/II trials, with the aim to stimulate strong functional T cell responses (4), capable of controlling viral replication or decreasing the HIV viral reservoir (5, 6). For the most part, these studies were disappointing, as they showed only modest effects on the control of viral replication after antiretroviral treatment interruption in HIV-infected individuals (7, 8). To counter the current lack of evidence of a potential benefit of

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The microarray data have been submitted to Gene Expression Omnibus under accession number GSE196172.

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Abbreviations used in this article: ANRS, French National Agency for Aids and Viral Hepatitis Research; FDR, false discovery rate; ICS, intracellular cytokine staining; mITT, modified intention-to-treat; MVA, modified vaccinia Ankara; SAE, serious adverse event; SFU, spot forming unit.

therapeutic vaccines for HIV functional cure (9), the therapeutic vaccine research field has remained active: >90 phase I/II trials are underway (10) to provide insights into potentially promising vaccine candidates or regimens based on favorable immune profiles.

In the prophylactic setting, the large phase III RV144 trial showed an encouraging but modest (31%) protective efficacy (11). However, 13 years later, the HVTN 702 trial (NCT02968849) testing the same vaccine platform (ALVAC vector plus the gp120 protein component) did not confirm these results and was stopped for lack of efficacy (12). Results of another ongoing phase IIb/III prophylactic trial (HVTN 705/HPX2008 study; NCT03060629) are pending. In the meantime, multiple prime-boost strategies involving different classes of vectors and HIV-1 protein combinations to induce broad and multifunctional T and B cell immune responses have been tested in preclinical and clinical trials (1, 13, 14) to select the best regimen to be moved into subsequent efficacy trials. The search for correlates of protection in RV144 suggests that it is important to generate both an Ab response recognizing the V1V2 V region of the HIV envelope glycoprotein (15) as well as T cell responses (16).

Taking into account the various vaccine candidates under development, as well as adjuvants and vaccine delivery systems, a multitude of prime-boost combinations is theoretically possible, and the number of vaccine strategies that needs to be downselected in early clinical development can become potentially very large. An additional level of complexity is related to the fact that the effects of heterologous prime-boost strategies on the immune system can be modulated by several factors, including the vaccine platforms themselves and their dose and route, as well as the number of injections and their administration sequence.

Besides the question of whether the prime-boost combination may impact the profile and the magnitude of vaccine-elicited immune responses, another important unanswered issue is whether the order of prime and boost platforms could influence the profile, magnitude, and durability of immune responses. This question has been rarely assessed in head-to-head evaluations within a single randomized multi-arm trial ensuring standardized methods and an unbiased evaluation across groups.

Thus, we hypothesized in this study that the order of the prime and boost components in a prime-boost strategy may be key for the induction of strong T cell responses. To address this, we designed a randomized proof-of-concept clinical trial to investigate whether a vaccine candidate given either as a prime or a boost is safe and impacts differently the profile, magnitude, and breadth of T cell responses. The design includes four heterologous prime-boost T cell vaccine strategies with three different vaccine candidates sharing similar HIV–T cell epitopes from HIV-1, that is, modified vaccinia Ankara (MVA) HIV-B (coding for Gag, Pol, Nef) (17) and HIV LIPO-5 (lipopeptides from Gag, Pol, Nef) vaccine (18, 19) given as a prime or a boost in prime-boost combination, or following GTU-MultiHIV B (DNA coding for Rev, Nef, Tat, Gag, Env gp160 clade B) (20, 21) priming. We further characterized immunogenicity with detailed assessments of T cell responses and whole-blood gene expression changes after vaccination in the different prime-boost combinations.

Materials and Methods

Ethics statement

The protocol was approved by an Ethics Committee (Comité de Protection des Personnes Ile-de-France V, Paris, France) and the competent French health authority (Agence Nationale de Sécurité du Médicament et des Produits de Santé [ANSM]) and was conducted in accordance with the Declaration of Helsinki. All volunteers provided written and signed informed consent for the trial. The trial was registered with ClinicalTrials.gov (NCT02038842) and EudraCT (2012-002456-17).

Vaccines

MVA virus is a highly attenuated strain of vaccinia virus. Characteristics of the French National Agency for Aids and Viral Hepatitis Research (ANRS) MVA HIV-B vector have been previously reported (17). Briefly, the vaccine was developed in collaboration with Transgene SA (Illkirch-Graffenstaden, France) based on a specific MVA isolate, MVATGN33. Recombinant HIV Ags include the full-length codon-optimized sequence of Gag (aa sequence 1–512) fused with fragments from Pol (aa 172–219, 325–383, 461–519) and Nef (aa 66–147, 182–206) from Bru/Lai isolate (Los Alamos accession number K02013). ANRS MVA HIV-B was administered at an i.m. dose of 0.5 ml (1×10^8 PFU/ml).

The ANRS HIV LIPO-5 vaccine candidate is an equal weight mixture of five synthetic lipopeptides. The peptide sequences are epitopes of the HIV Gag, Pol, and Nef proteins of the HIV-1 clade B virus (Gag 17–35 and 253–284, Nef 66–97 and 116–145, Pol 325–355). The production of the individual lipopeptides was performed by Bachem (Bubendorf, Switzerland), and the manufacturing of HIV LIPO-5 (from the individual lipopeptides) was performed by Baccinex (Courroux, Switzerland). HIV LIPO-5 was administered at an i.m. dose of 1 ml (2.5 mg/ml).

The investigational HIV-1 vaccine GTU-MultiHIV B clade was developed by FIT Biotech (Tampere, Finland). It encodes for a MultiHIV Ag (synthetic fusion protein built up by full-length polypeptides of Rev, Nef, Tat, and Gag p17 and p24 with >20 Th and CTL epitopes of protease, reverse transcriptase, and gp160 regions of the HAN2 HIV-1 B clade. This vaccine was administered at a dose of 1 ml (1 mg/ml), combining i.m. (0.5 ml, with the Biojector 2000 needle-free device) and intradermal (0.5 ml) routes.

Study design

The ANRS VRI01 trial was an open-label, multicenter, randomized phase I/II trial with four parallel groups conducted in four sites in France. INSERM-ANRS was the sponsor of the trial. Volunteers meeting the eligibility criteria were randomly allocated in a 1:1:1:1 ratio at trial entry to receive one of the following open-label HIV vaccine strategies: 1) MVA HIV-B at weeks 0 and 8, and HIV LIPO-5 at weeks 20 and 28 (group M/L); 2) HIV LIPO-5 at weeks 0 and 8, and MVA HIV-B at weeks 20 and 28 (group L/M); 3) GTU-MultiHIV B at weeks 0, 4, and 12, and HIV LIPO-5 at weeks 20 and 28 (group G/L); and 4) GTU-MultiHIV B at weeks 0, 4, and 12, and MVA HIV-B at weeks 20 and 28 (group G/M) (Fig. 1). The trial included a phase I safety assessment of MVA HIV-B as well as a phase II immunogenicity assessment in each of the four groups (Fig. 1). This trial was designed for statistical within-group comparisons (noncomparative randomized design with a Fleming design per group). Detailed methodological considerations of this design have been published previously (22).

Study participants

Prior to screening all volunteers provided written consent to participate in the trial. Volunteers were eligible when aged between 21 and 50 y and at low risk of HIV infection. Volunteers were excluded when they had any relevant abnormality on medical history or during clinical or laboratory examination, a history of severe local or general reaction to vaccination, had received a live attenuated vaccine within 60 d or another vaccine within 14 d prior to the first vaccination, had received recent blood products or Ig, or had previously participated in a HIV vaccine clinical trial. During follow-up visits, solicited and unsolicited adverse events were recorded, and blood samples were drawn from immunogenicity assessments. Immunogenicity assessments were performed in batch at the end of the trial in a centralized laboratory (VRI/INSERM U955 immunomonitoring platform; MIC-VRI, Creteil, France).

Randomization and masking

The randomization list was generated centrally by a statistician at the Clinical Trials Unit (INSERM U1219, University of Bordeaux) in a 1:1:1:1 ratio with SAS software, using permuted block sizes without stratification factors. The list was implemented into an electronic case report form–based randomization tool (Ennov Clinical) that was then used by the site investigators to randomize the volunteer while he or she was present on site for the first vaccination visit (baseline visit [week 0]).

Clinicians and volunteers were not blinded to the allocated vaccine strategies, but members of the Endpoint Review Committee (review of relevant clinical adverse events) were blinded. The central immunogenicity laboratory assessed study samples blinded to randomized group and to study visit.

Immunological evaluation

IFN-γ ELISPOT assay. The immunogenicity of vaccines was assessed by the quantification of T cell responses, that is, spot-forming units (SFU), using an IFN-γ ELISPOT assay following the manufacturer's instructions (human IFN-γ ELISpot^{PRO} kit, Mabtech) against a panel of HIV overlapping peptides (15-mers with 11-aa overlap, $n = 276$, JPT Peptide Technologies, Berlin,

Germany) spanning the whole sequences of Gag/Tat/Rev proteins and partial sequences of Pol/Env/Nef proteins, grouped in 15 pools (Gagp17-1, Gagp17-2, Gagp24-1, Gagp24-2, Gagp24-3, Gagp2/p6-1, Gagp2/p6-2, Pol/Env, Pol-2, Pol-3, Nef-1, Nef-2, Nef-3, Tat, and Rev). Assays were performed in a centralized laboratory on cryopreserved PBMCs at baseline (week 0) and 2 wk after each vaccine injection (weeks 2, 10, 22, and 30 for groups M/L and L/M, or weeks 2, 6, 14, 22, and 30 for groups G/L and G/M).

Intracellular cytokine staining assay. To assess Ag-specific T cell responses, an intracellular cytokine staining (ICS) assay was performed on cryopreserved PBMCs. PBMCs were rested overnight and then stimulated (6h, 37°C, 5% CO_2) with four peptide pools (Gag, Pol/Env, Nef, and Tat/Rev, 2μg/ml) in the presence of anti-CD28 and anti-CD49d Abs (1 μg/ml each) and GolgiPlug (10 μg/ml) (BD Biosciences, Le Pont de Claix, France). Staphylococcal enterotoxin B stimulation (100 ng/ml staphylococcal enterotoxin B; Sigma-Aldrich, Saint Quentin Fallavier, France) served as a positive control. After stimulation, cells were stained for dead cells with an amine-reactive dye (LIVE/DEAD Aqua, Invitrogen, Life Technologies, Saint Aubin, France) for 20 min at room temperature, washed, and labeled with fluorochrome-conjugated mAbs (anti-CD3 Alexa Fluor 700, anti-CD4 PE, and anti-CD8 allophycocyanin-H7; all from BD Biosciences) for 15 min at room temperature. After fixation and permeabilization using a Cytofix/Cytoperm kit (BD Biosciences) for 20 min and staining with anti–IFN-γ PerCP-Cy5.5, anti–TNF-α PE-Cy7, and anti–IL-2 allophycocyanin (all BD Biosciences) for 20 min at room temperature, PBMCs were resuspended in BD CellFIX (BD Biosciences) and stored at 4°C until analysis. Data were acquired on a LSRFortessa four-laser (488, 640, 561, and 405 nm) flow cytometer (BD Biosciences). At least 250,000 events gated on $CD3^+$ were collected and analyzed with FlowJo software, version 9.9.4 (Tree Star).

Gene expression analysis

Total RNA was purified from whole blood using the Tempus spin RNA isolation kit (Thermo Fisher Scientific). RNA was quantified using an ND-8000 spectrophotometer (NanoDrop Technologies, Fisher Scientific, Illkirch-Graffenstaden, France) and quality control performed on a 2100 Bioanalyzer (Agilent Technologies, Massy, France). cDNA was synthesized and biotin-labeled cRNA was generated by an in vitro transcription reaction using Ambion Illumina TotalPrep RNA amplification kits (Applied Biosystems/Ambion, Saint-Aubin, France). Labeled cRNA was hybridized on Illumina human HT-12V4 BeadChips according to the Illumina protocol. The beads were then read on an iScan Illumina system. Raw data generation and quality controls were performed using GenomeStudio Illumina software.

All microarray data are MIAME (minimum information about a microarray experiment) compliant, and the raw and normalized data have been deposited in the MIAME-compliant database Gene Expression Omnibus (GEO series accession number GSE196172, http://www.ncbi.nlm.nih.gov/geo/). Canonical pathway and biological function analyses were carried out with the Ingenuity Pathway Analysis software (QIAGEN, https://digitalinsights.qiagen.com/products-overview/discovery-insights-portfolio/analysis-and-visualization/qiagen-ipa/) using genes differentially expressed between groups with false discovery rate (FDR)–adjusted $p \leq 0.05$.

Study endpoints

Because the ANRS MVA HIV-B vaccine candidate was used for the first time in humans in this trial, the MVA HIV-B co-primary safety endpoint was defined as the proportion of participants without any grade 3 or 4 adverse events (clinical or biological) related to MVA vaccine immunization reported from week 0 to week 2 in group M/L.

The co-primary immunogenicity endpoint was defined as the proportion of participants with an HIV-specific IFN-γ ELISPOT response at week 30 in each group, that is, 2 wk after the last vaccine injection. An HIV-specific IFN-γ ELISPOT response was defined by a positive response to at least one of the stimulating HIV peptides with a mean SFU across replicate wells ≥55 SFU/10^6 cells and ≥4-fold the negative control. Response to an HIV peptide pool was only considered positive after vaccination if not already positive at baseline.

Secondary endpoints included adverse events throughout the whole follow-up period in each trial group, the magnitude of HIV-specific IFN-γ ELISPOT and of ICS responses, and changes in gene expression 2 wk after each injection. ICS responses were assessed only in groups having received the MVA HIV-B vaccine.

Statistical analysis

The sample size was calculated for a single-stage Fleming design within each trial group: for the co-primary immunogenicity endpoint measured at week 30 in each group, we targeted a level (p1) of 80% ELISPOT responders, and aimed at rejecting the vaccine strategy for insufficient immunogenicity in the case of ≤50% ELISPOT responders (p0). With one-sided $\alpha = 5\%$ and 90% power, 23 participants needed to be assessed in each group (23). The given prime-boost strategy was considered to be immunogenic enough to proceed to further clinical development when the lower bound of the one-sided 95% confidence interval of the proportion of ELISPOT responders at week 30 was >50%.

Continuous safety monitoring with repeated interim analyses was implemented for ANRS MVA HIV-B, which was administered for the first time in humans in this trial. The continuous monitoring of the co-primary safety endpoint was performed in a Bayesian statistical framework in the participants randomized to group M/L and having received at least one dose of MVA HIV-B (22). In final analyses, a frequentist framework was used in addition to the Bayesian framework to describe the safety of MVA HIV-B.

The co-primary immunogenicity endpoint and the secondary immunogenicity endpoints were analyzed per group, in a frequentist framework, in the modified intention-to-treat (mITT) population (all participants as randomized, having received at least one vaccine injection and still HIV-negative at week 30) as well as in the per-protocol population (all randomized participants having received all vaccine administrations initially assigned by the randomization and with available samples for all immunogenicity time points, that is, five time points in groups M/L or L/M and six time points in groups G/L or G/M, and still HIV-negative at week 30).

The number and proportion (with 95% one-sided confidence interval) of participants with an HIV-specific IFN-γ ELISPOT response at week 30 (co-primary immunogenicity endpoint) were described for each group. When the lower confidence limit was >50%, then the vaccine strategy of the given group was considered interesting for the future clinical development. The magnitude of HIV-specific IFN-γ ELISPOT responses 2 wk after each vaccine immunization was analyzed as the mean number of spot-forming cells/10^6 PBMCs across replicate wells. For the overall response of each participant, SFU were added up across 15 HIV peptide pools (assuming that there is no overlap in response across peptide pools), per participant and time point, after removing background noise.

ICS responses were analyzed after subtracting background values from nonstimulated samples, with negative values put to zero. Within-group comparisons were performed in each group using Wilcoxon signed-rank tests. We used a FDR method to adjust for test multiplicity for dependent comparisons (24) after HIV stimulation between week 0 and week 30, which was the time point of primary interest. Comparisons at other time points or individual peptide stimulations were not multiplicity adjusted.

Gene transcription data were preprocessed (25, 26) and corrected for a potential batch effect (27). Statistical analyses for time course gene expression analyses were performed using mixed-effects regression models with a random intercept and random slopes, and a variance component score test for significance testing with FDR adjustment for multiplicity using dearseq for gene-wise analysis (28) and time-course gene set analysis (TcGSA) for gene set analyses (29). Chaussabel modules and blood transcriptional modules were used as gene set definitions (30, 31). The three randomized groups receiving a vaccine strategy containing HIV MVA-B as prime or boost were pooled and the specific effect of HIV MVA-B was tested, including a (non-significant) test for interaction with the group effect in the model.

Analyses were performed using SAS software (SAS Institute, Cary, NC; version 9.3 or higher) and R software (the R Foundation, Vienna, Austria; version 3.5).

Results

Recruitment and follow-up

From March 2014 to March 2015, 129 participants were screened in four sites in France, and 92 were randomized in a 1:1:1:1 ratio to receive one of the following open-label HIV vaccine strategies in this phase I/II multiarm trial: 1) MVA HIV-B at weeks 0 and 8, and HIV LIPO-5 at weeks 20 and 28 (group M/L); 2) HIV LIPO-5 at weeks 0 and 8, and MVA HIV-B at weeks 20 and 28 (group L/M); 3) GTU-MultiHIV B at weeks 0, 4, and 12, and HIV LIPO-5 at weeks 20 and 28 (group G/L); and 4) GTU-MultiHIV B at weeks 0, 4, and 12, and MVA HIV-B at weeks 20 and 28 (group G/M) (Fig. 1). The ANRS MVA HIV-B was tested for the first time in humans in this trial.

All randomized participants received at least one vaccine injection (Fig. 2). Baseline demographics were similar between the four randomized groups: 54% of the participants were male, and the median age was 27 y. Overall, 91 of 92 participants completed all vaccine injections.

Safety

Ninety-two volunteers were included in the safety analysis. Local and systemic reactogenicity events were frequent (87–100% of volunteers)

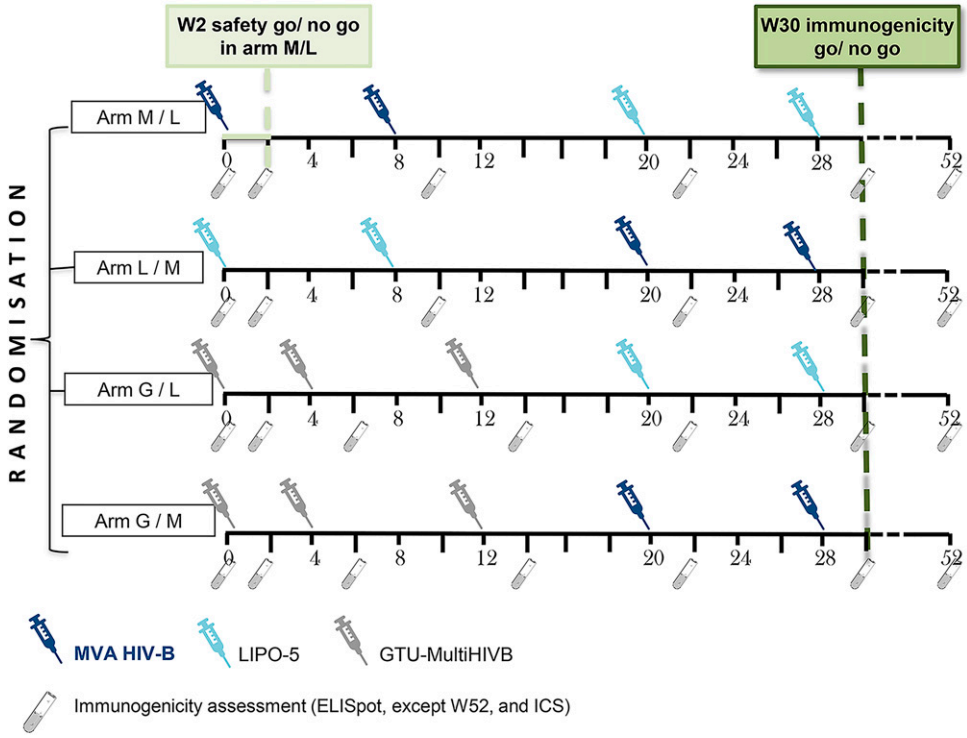

FIGURE 1. Overview of the ANRS VRI01 trial design. W, week.

in all groups but limited to grade 1 and 2 events. Throughout the trial, 28 serious adverse events (SAEs) were reported, among which 4, 0, 7, and 1 SAEs were possibly related to vaccination in groups M/L, L/M, G/L, and G/M, respectively, including one case of myelitis possibly related to HIV LIPO-5 (Table I). The other SAEs possibly related to vaccination were vitiligo leading to vaccine discontinuation (group G/L), epilepsy (group G/L), and biological events (groups M/L, G/L, and G/M). Only one participant from group M/L reported

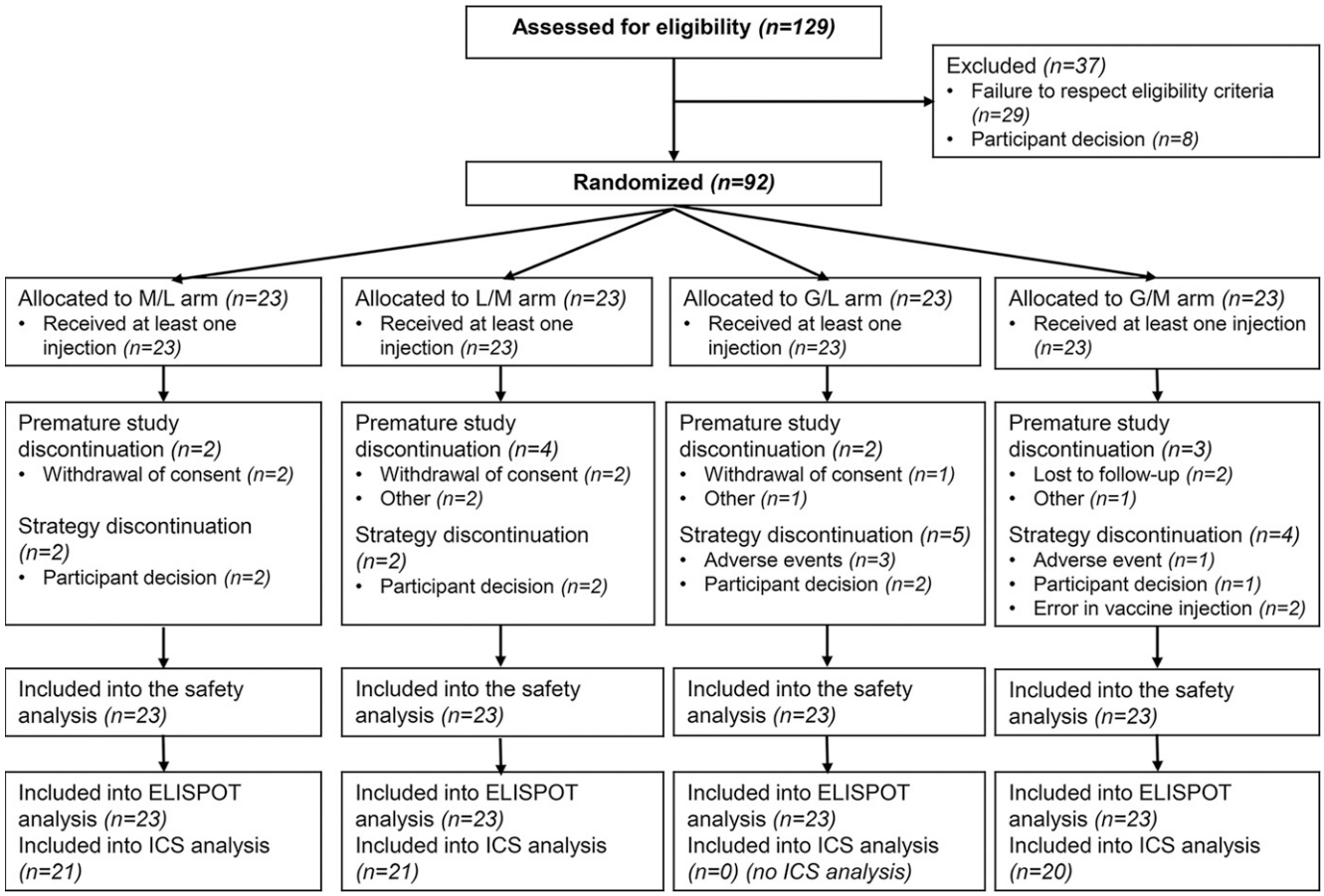

FIGURE 2. Flow diagram of participant enrollment and follow-up.

Table I. Adverse events per randomized group

	Group M/L (n = 23)		Group L/M (n = 23)		Group G/L (n = 23)		Group G/M (n = 23)		Total (n = 92)	
Participant with at least one grade 3 or 4 AE, n (%)	3	(13.0)	5	(21.7)	5	(21.7)	3	(13.0)	16	(17.4)
Total number of grade 3 or 4 AEs, n	8		7		8		3		26	
Total number of grade 3 or 4 AEs related to vaccine immunization,[a] n	4		0		5		1		10	
Participant with at least one AE related to vaccine immunization leading to discontinuation, n (%)	0	(0.0)	0	(0.0)	1	(4.3)	0	(0.0)	1	(1.1)
Total number of AE related to vaccine immunization leading to discontinuation,[b] n	0		0		1		0		1	
Participant with at least one SAE, n (%)	3	(13.0)	5	(21.7)	7	(30.4)	3	(13.0)	18	(19.6)
Total number of SAEs, n	8		7		10		3		28	
Total number of SAEs related to vaccine immunization,[c] n	4		0		7		1		12	

Relatedness to immunization according to investigator and/or Endpoint Review Committee and/or sponsor pharmacovigilance department. AE, adverse event; SAE, serious AE.

[a] Anemia, lipase increased (n = 3), hemoglobin decreased (n = 2), protein urine present (n = 2), blood creatine phosphokinase and lipase increased, myelitis.

[b] Vitiligo.

[c] Anemia, vitiligo, lipase increased (n = 3), hemoglobin decreased (n = 2), protein urine present (n = 2), blood creatine phosphokinase and lipase increased, epilepsy, myelitis. Among 12, 5 were considered as study vaccine related by investigators and/or sponsor.

a grade 3 adverse event (lipase increase to 263 IU/ml and creatine phosphokinase increase to 1343 IU/ml after physical efforts) from week 0 to week 2 that was judged related or possibly related to MVA HIV-B by the Endpoint Review Committee, but judged to be unrelated by the investigator and the sponsor. Regardless of the time point in groups M/L, L/M, and G/M, two participants (both in group M/L) had grade 3 adverse events (a lipase and creatine phosphokinase increase and a hemoglobin increase) related or possibly related to MVA HIV-B according to the Endpoint Review Committee (but judged unrelated by the investigator and by the sponsor). Bayesian safety analysis results of MVA HIV-B, using a decision rule based on Bayesian posterior probabilities (22), indicated no safety concern.

T cell immunogenicity

Ninety-two volunteers were included in the T cell response analysis assessed by IFN-γ ELISPOT. Two weeks after the prime (i.e., week 10 in M/L and L/M and week 14 in both G/L and G/M groups) the proportion of IFN-γ ELISPOT responders and median SFU/10^6 PBMCs among responders were 59% (328), 5% (323), and 0% in M/L, L/M, and combined G/L and G/M groups, respectively (mITT analysis).

Two weeks after boost injections, at week 30 (primary endpoint, per-protocol immunogenicity analysis), these proportions and median number of SFU/10^6 PBMCs were 33% (250), 43% (528), 0%, and 74% (324) in M/L, L/M, G/L, and G/M groups, respectively (Fig. 3). This trial was designed to compare the observed proportion of responders at week 30 within each group to a predefined minimum immunogenicity level of 50%: in the per-protocol population the proportion of responders in the G/M group was significantly above the predefined minimum immunogenicity level (p = 0.02 for superiority to 50% threshold). In mITT analyses, this was at the limit of significance (67%, p = 0.06 for superiority to 50% threshold).

To further characterize T cell responses to the vaccine strategies, we performed intracellular cytokine staining responses in the per-protocol population of the three groups that received MVA HIV-B in the randomized prime-boost strategy (n = 62), while the G/L group was discarded from further analyses due to the absence of IFN-γ ELISPOT response. The percentages of HIV-specific $CD4^+$ T cells producing at least one cytokine (out of IFN-γ, IL-2, TNF-α) after stimulation by HIV peptides increased from week 0 to week 30 in M/L, L/M, and G/M groups (median change of 0.06, 0.11, and 0.10%, respectively; FDR-adjusted $p < 0.001$ within each group), mainly after MVA HIV-B vaccinations (Fig. 4, Supplemental Fig. 1). This was sustained until week 52 (unadjusted p = 0.03, <0.001, and <0.001 for week 0 versus week 52 comparison in M/L, L/M, and G/M groups, respectively).

HIV-specific $CD8^+$ T cell responses increased from week 0 to week 30 in M/L and G/M groups (median change of 0.02%, FDR-adjusted p = 0.01, and 0.05%, FDR-adjusted $p < 0.001$, respectively), but they were not significantly sustained at week 52.

Analysis of the polyfunctionality of HIV-specific T cells showed at week 30 a high frequency of cells producing mainly IFN-γ and TNF-α in the three groups, which persisted at week 52, that is, 24 wk after the last boost (Fig. 5, Supplemental Fig. 1). At this later time point, polyfunctional (producing IL-2/TNF-α/IFN-γ) and polyepitopic $CD4^+$ T cells redirected against Gag Ag, with additional responses to Pol/Env and Nef, were significantly detectable in the L/M and G/M groups, whereas $CD8^+$ T cell responses (producing TNF-α, IFN-γ) were present only in the L/M group (Fig. 6, Supplemental Table I).

Changes in gene expression after MVA HIV-B injections

Whole-blood gene expression changes after MVA HIV-B injections were assessed by microarray and analyzed using a modeling approach taking into account data from the three randomized groups having received this vaccine. This resulted in an MVA HIV-B gene signature including 86 genes (96 probes) that varied significantly after the first MVA injection regardless of its administration as a prime or a boost (Supplemental Table II). Fig. 7A shows the dynamics of the 96 probes in each randomized group, and Fig. 7B shows the gene expression levels of the subset of 21 significant probes with fold change >1.5 per time point and group. The significant probes, such as those encoding for the cell division cycle protein 20 homolog (*CDC20*), the cell division cycle–associated protein 5 (*CDCA5*), and the cell division cycle–associated protein 7 (CDCA7), were mainly related to cell cycle functions and pathways (Fig. 8, Table II).

In the statistical models, adjusted for group, time point, and type of vaccine, no prime or boost signal independent from the observed MVA HIV-B signal was detected. The results also did not show any significant signals of changes in gene expression after the second MVA HIV-B injections. No significant gene expression changes were detected in the G/L group.

We also analyzed the MVA HIV-B signature in gene set analyses, using module definitions instead of analyses per individual microarray probe in the statistical models. Using Chaussabel modules (30), we found 76 modules that varied significantly after MVA HIV-B injections. These included modules for inflammation, that is, cell cycle, T cells, cytotoxic/NK cells, monocytes, and plasma cells

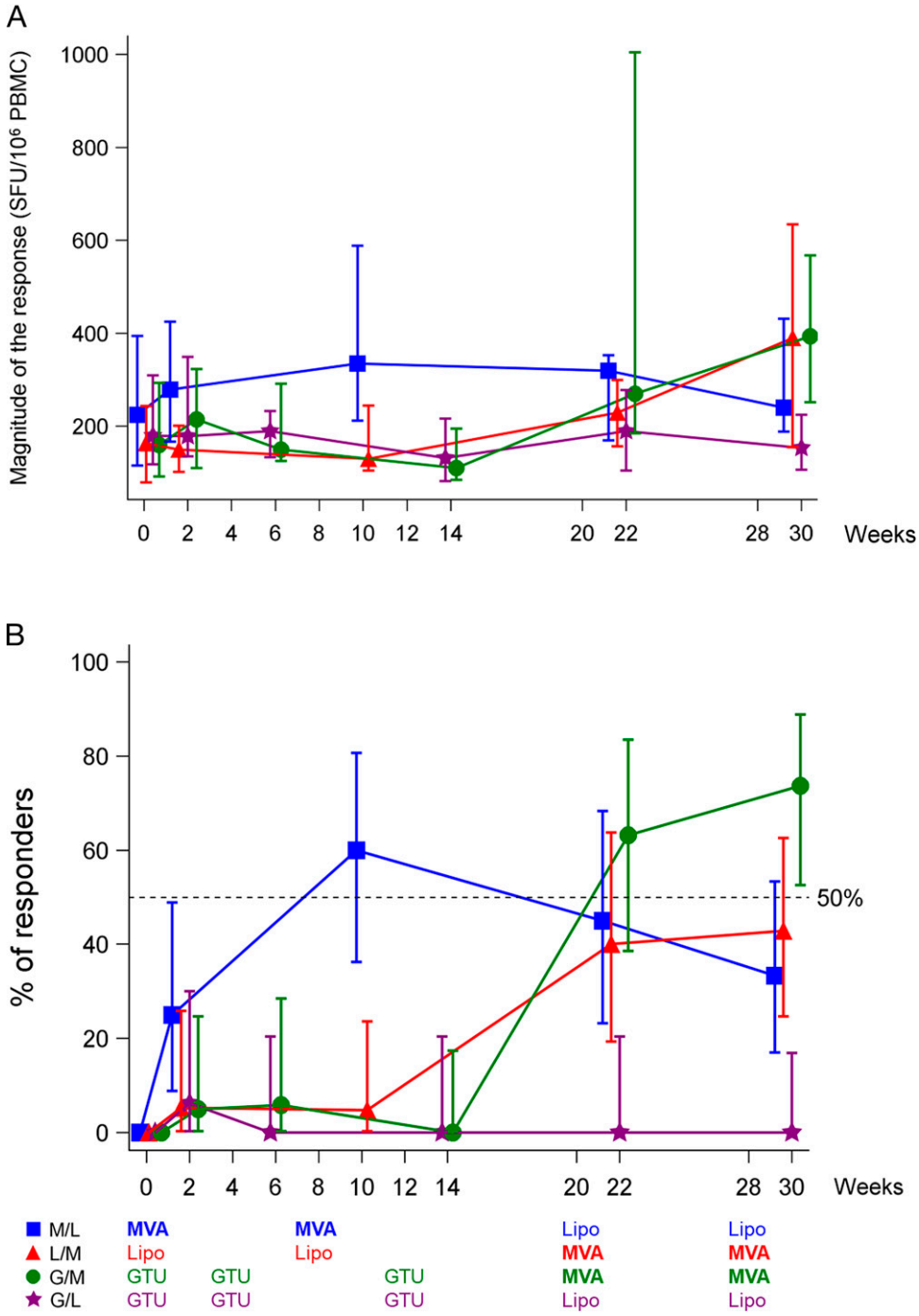

FIGURE 3. IFN-γ ELISPOT responses per randomized group and time point (per-protocol immunogenicity analysis). (**A**) Magnitude of response (SFU/10^6 PBMCs) of all volunteers of the per-protocol population, per group and time point. Median and interquartile range. (**B**) Proportion of responders. Two-sided 95% confidence interval for weeks 0, 2, 6, 14, and 22 (secondary endpoints) and two-sided 90% confidence interval for week 30 (primary endpoint).

(Supplemental Table III). Using blood transcriptional modules (31), the number of modules varying significantly after MVA HIV-B injections was 59, covering cell cycle modules but also modules annotated for immune functions and inflammation (Supplemental Table IV).

We further looked for the enrichment of a specific MVA HIV-B signature that was previously reported from an in vitro experimentation with single-cell gene expression analyses (single-cell RNA sequencing) of dendritic cells and that had defined three gene expression clusters (32). We found that two of these three clusters previously identified in vitro were also enriched in our clinical trial data after MVA HIV-B injection (cluster 0, named "bystander activation of dendritic cells" by the authors of the in vitro study, p value 0.007; and cluster 2, named "activated and infected dendritic cells," p value 0.0007).

Discussion

Early clinical vaccine development is usually a lengthy process requiring successive phase I and II trials. In an effort to optimize early-phase HIV-1 vaccine trials and the methods to downselect the most promising vaccine strategies, we used an innovative randomized multiarm trial design (22). The overall objective was to discard unpromising vaccine regimes following a head-to-head comparison of four heterologous prime-boost strategies. This allowed to assess three vaccines (i.e., either HIV LIPO-5, DNA GTU-MultiHIV B, or MVA HIV-B) tested as a prime and, in a reverse order, as a boost (HIV LIPO-5 or MVA HIV-B).

We showed that the MVA HIV-B vaccine used as a prime (two injections) was more potent, leading to a higher frequency of T cell ELISPOT responders (close to 60%) than HIV LIPO-5 (two

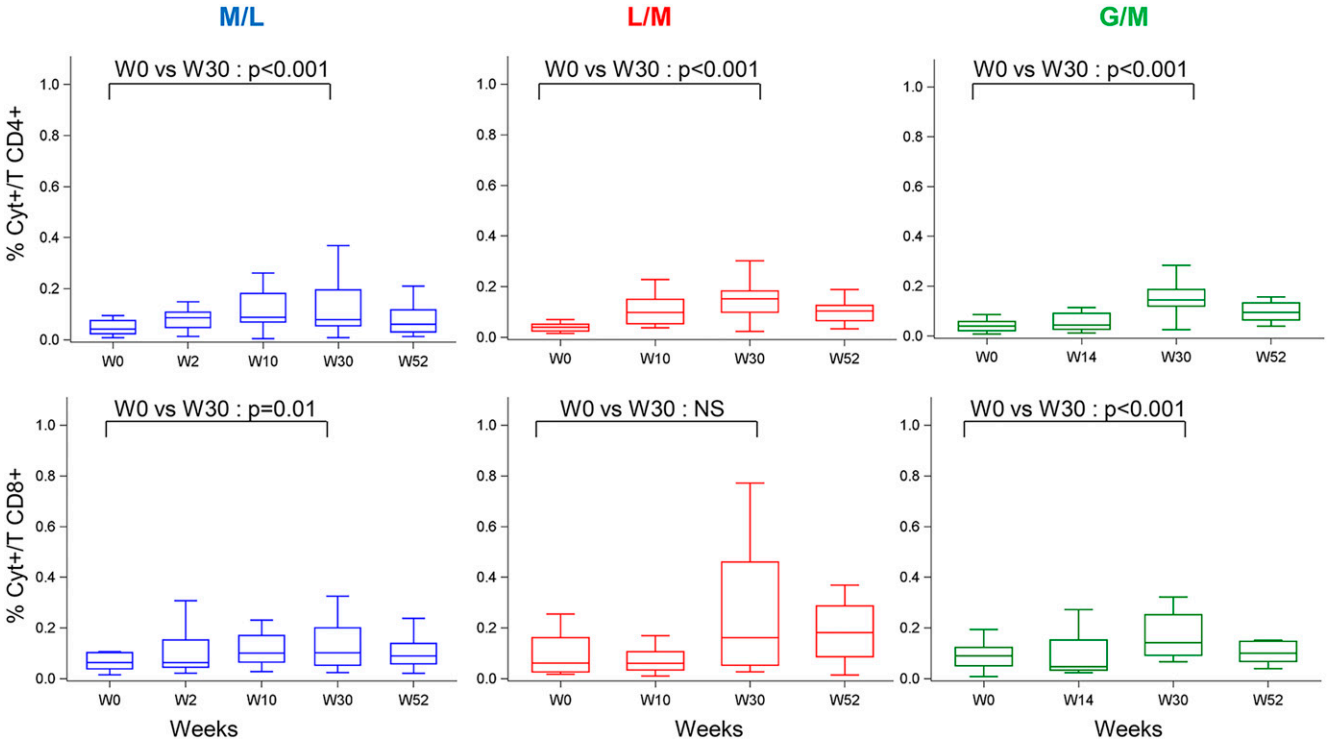

FIGURE 4. Percentages of $CD4^+$ and $CD8^+$ T cells producing at least one cytokine after HIV stimulation, assessed by intracellular cytokine staining per randomized group and time point (per-protocol immunogenicity analysis). Boxes show the median and interquartile range (IQR), and whiskers show 1.5-fold the IQR. FDR-adjusted *p* values for week 30 to week 0 comparisons. Comparisons between week 52 and week 0 were exploratory without FDR adjustment, and *p* values are thus not shown on the figure. W, week.

injections; 5%) or DNA GTU MultiHIV (three injections 0%). This latter disappointing observation was consistent in the two groups testing DNA GTU MultiHIV prime (G/L and G/M). When tested as a boost, MVA HIV-B improved the frequency of ELISPOT responders in groups primed either with HIV LIPO-5 (43%) or DNA GTU MultiHIV (74%), whereas no increase in the rate of responders was observed following HIV LIPO-5 boost. Moreover, we showed that the frequency of polyfunctional vaccine-specific $CD4^+$ T cells was sustained 6 mo after the last boost in M/L, L/M, and G/M groups. Finally, we characterized an MVA HIV-B gene expression signature marked by changes in the abundance in the expression of genes belonging to cell cycle pathways. These changes were present when

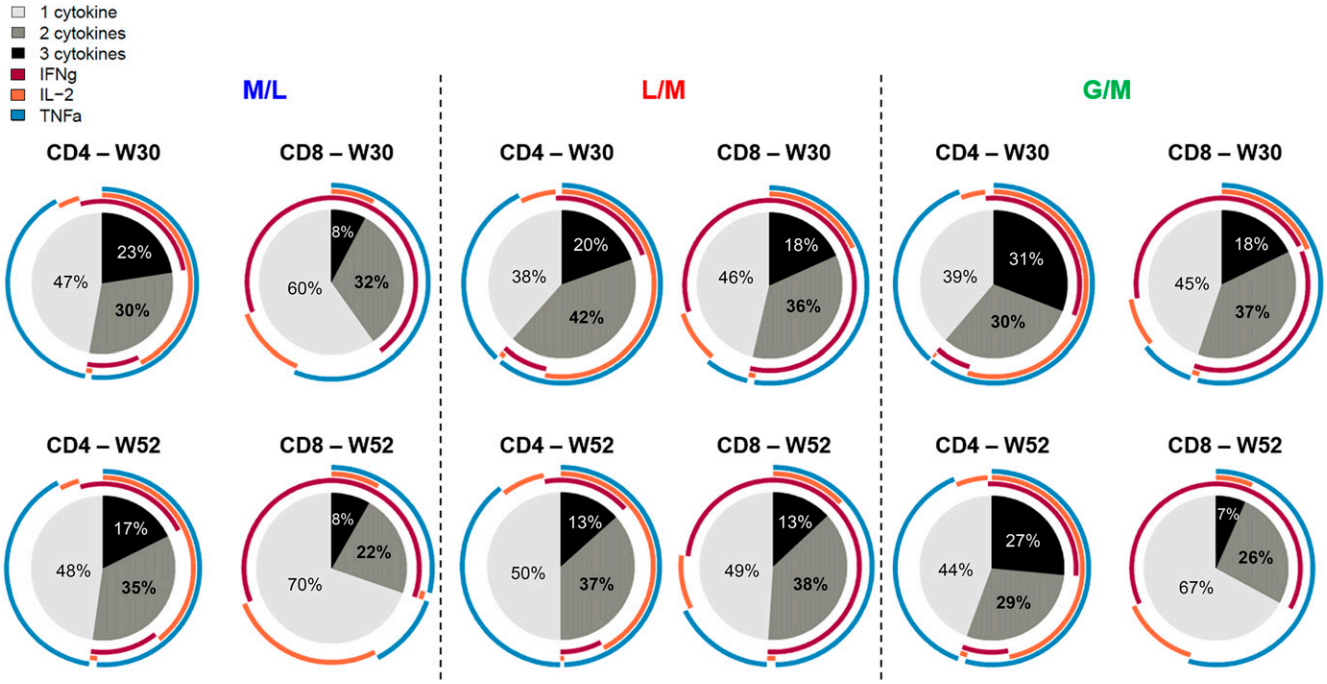

FIGURE 5. Polyfunctionality of $CD4^+$ and $CD8^+$ T cells after HIV stimulation, assessed by intracellular cytokine staining per randomized group at week 30 and week 52 (per-protocol immunogenicity analysis). W, week.

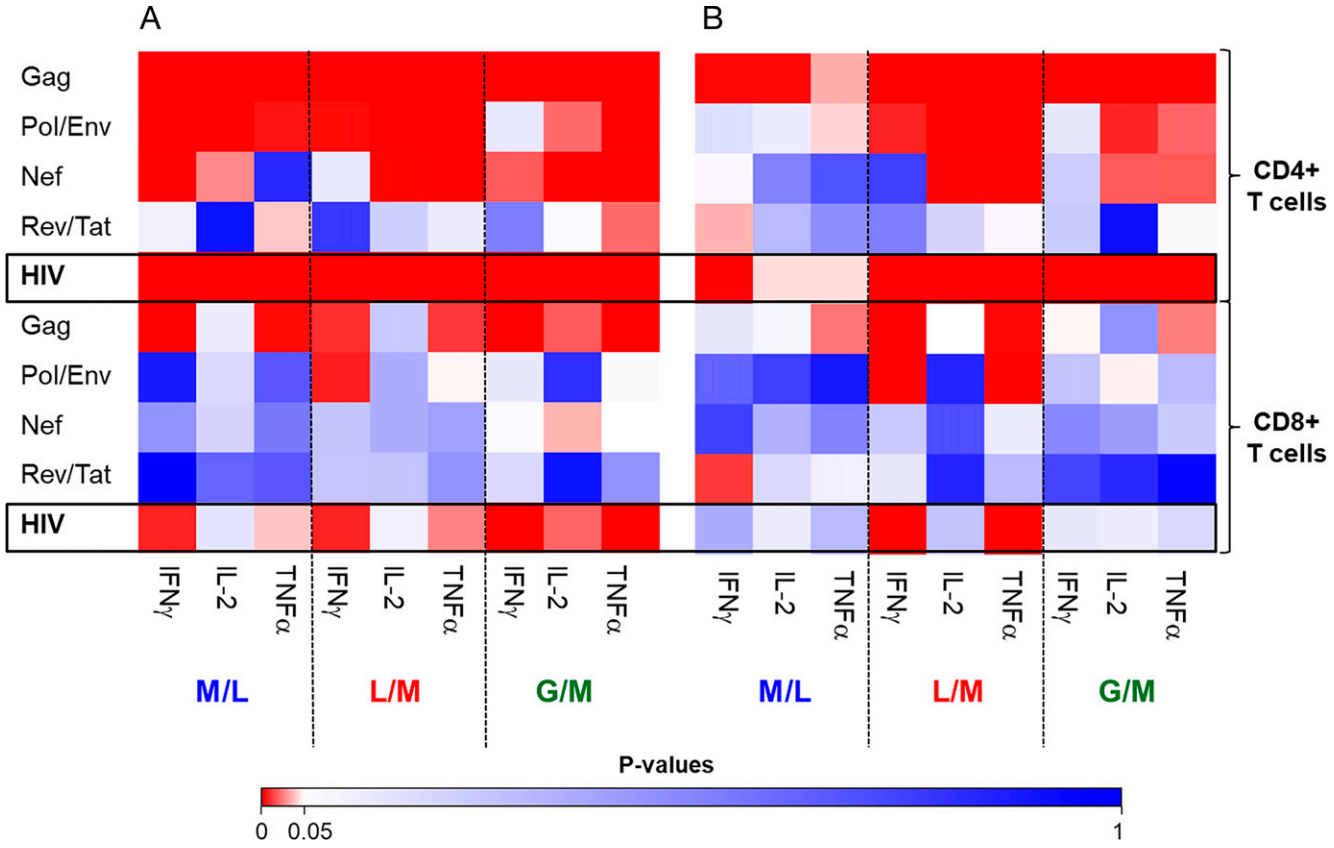

FIGURE 6. Heatmap of *p* values for comparisons of frequency of cytokine-positive $CD4^+$ and $CD8^+$ T cells, assessed by intracellular cytokine staining per randomized group and stimulating peptide pool (per-protocol immunogenicity analysis). (**A**) Raw *p* values for comparisons between week 0 and week 30. (**B**) Raw *p* values for comparisons between week 0 and week 52.

the MVA HIV-B vaccine was administered either as a prime or a boost, suggesting the existence of a consistent signature of MVA HIV-B.

Globally, according to a predefined selection criterion, the experimental design of this study allowed us to rapidly identify the G/M combination as the best strategy inducing a higher frequency of T cell responders. Moreover, we demonstrated that the order of administration of a vaccine, as a prime or a boost, may influence the quality, magnitude, and durability of T cell responses.

Our results extended previous studies showing that the combination of HIV vaccines and the frequency of injections in prime-boost strategies may influence the rate and nature of immune responses (33–35). As a few recent examples, we have previously shown that an HIV-DNA-C HIV-NYVAC-C prime-boost combination is highly immunogenic and that a better priming of poxvirus-based vaccine regimens for T cells is obtained with three DNA injections, while humoral responses were higher with two DNA-C primes (33). The phase I HVTN 111 trial showed that prime-boost or coadministration of HIV subtype C DNA and MF59-adjuvanted subtype C Env protein elicited variable anti-V1/V2 Abs and $CD4^+$ T cell responses (36). Moreover, the mode of delivery of the DNA vaccine (i.e., Biojector or syringe) influenced the magnitude of $CD4^+$ T cell responses in the prime-boost regimen but not in the coadministration regimen (36). In another study, coadministration of gp120 Env protein during the priming (either with DNA or NYVAC vectors) led to early and potent induction of Env V1/V2 IgG binding Ab responses and better and sustained Ab response coverage than did delayed administration of gp120 Env protein (35).

Our results might help to design future proof-of-concept prophylactic and therapeutic vaccine trials. We have focused on the analysis of responses induced by "T cell vaccines" that could be of interest for the development of both types of vaccine strategies (37). Although the main objective in the prophylactic vaccine field is to design strategies to elicit protective HIV-1–specific responses through the induction of broadly neutralizing Abs, induction of potent and efficient T cell responses remains important and still challenging (4). The role of T cells in protection against HIV acquisition was disputed following the failure of the STEP and the HVTN 505 trials (38–41). Nevertheless, in the latter, higher T cell responses induced by a DNA/rAd5 HIV-1 preventive vaccine strategy were associated with lower HIV-1 infection risk (42). In other HIV vaccine trials, the functional profile of vaccine-induced T cells has been associated with a reduced risk of HIV infection (16). Moreover, Env-specific $CD4^+$ T cell responses have been identified as one correlate of protection in vaccinated and uninfected volunteers in the RV144 trial (15). Finally, an association between functional and robust T cell responses and protection is widely suggested by several recent animal challenge studies using viral vectors (43–45).

In the therapeutic HIV vaccine field, some studies have suggested an association between the functional profile of vaccine-elicited T cell responses and a partial control of HIV replication following antiretroviral interruption in vaccinees (46–50). However, in most trials, an efficient control of HIV replication could not be established despite T cell responses. Several reasons have been evoked to explain these disappointing results, including the exhaustion of the immune system, the lack of efficient killing of $CD8^+$ T cells, or a limitation of the breadth of T cell responses (5, 7). The vaccine candidates tested in our present study were selected on the basis of sharing a large number of T cell epitopes delivered through

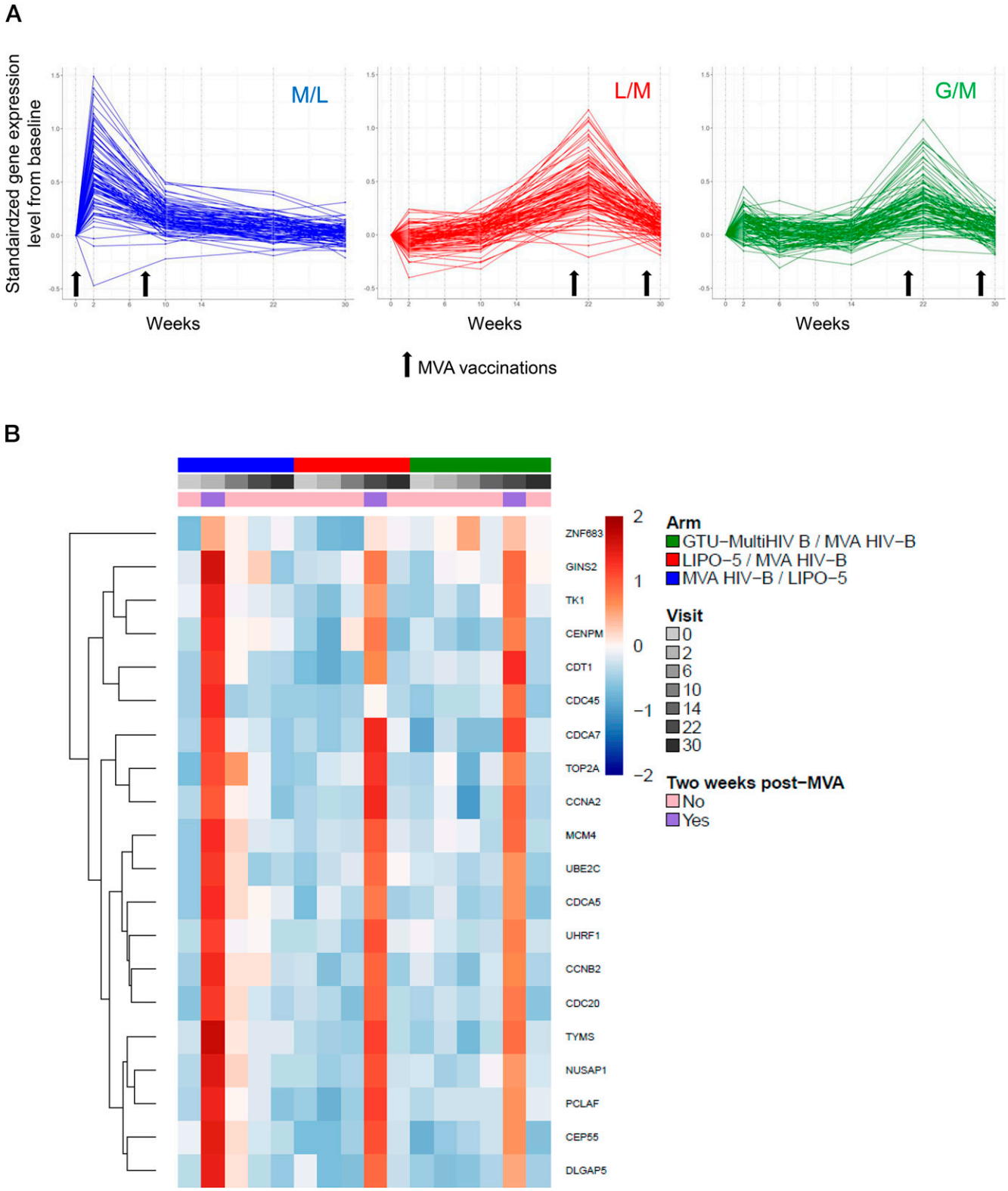

FIGURE 7. Changes in whole-blood gene expression after HIV MVA-B vaccination as prime or boost (per-protocol immunogenicity analysis). (**A**) Each plot corresponds to a randomized group receiving the HIV MVA-B vaccination. Black arrows indicate the timing of HIV MVA-B injections. Each line in the plots corresponds to one of the 96 significantly varying probes (mean expression). (**B**) Heatmap of median standardized gene expression per time point in each randomized group receiving the HIV MVA-B vaccination. Genes that are significantly differentially expressed 2 wk after the first MVA injection (time point indicated in purple in the upper bar) with fold change >1.5 are shown (20 genes).

heterologous strategies. However, one caveat of this approach could be raised following a recent randomized clinical study suggesting a risk of an antigenic competition (notably between Gag and Env) when multiple Ags are contained in the vaccines (51). This could lead to a reduced breadth of T cell responses and the lack of viral control (52). Although arguments for this mechanism are limited in

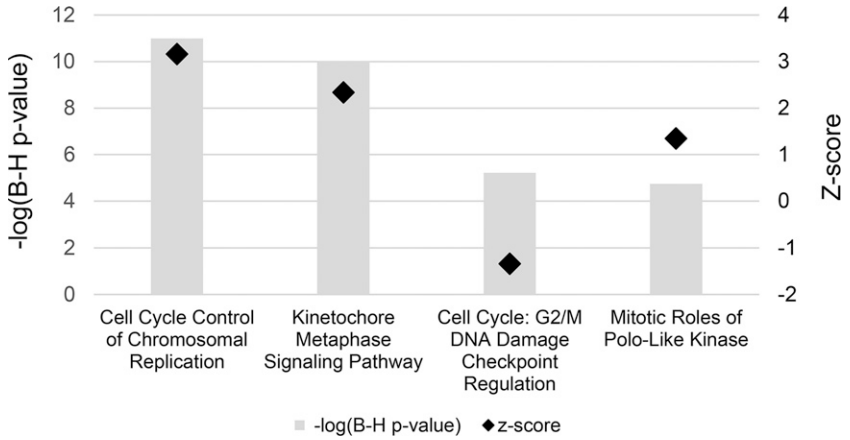

FIGURE 8. Main enriched canonical pathways after HIV MVA-B vaccination as prime or boost. Ingenuity pathway analyses using the 96 significantly varying probes after HIV MVA-B vaccination. *Z* score ≥2 was defined as the threshold significant activation, while a *Z* score of −2 or less was defined as the threshold of significant inhibition of a canonical pathway. B-H *p* value, Benjamini–Hochberg multiplicity adjusted *p* value.

humans, inhibition of vaccine-elicited Gag-specific cellular responses in the presence of increasing doses of Env has been shown in a nonhuman primate model (53).

Of note, the G/L group tested in the current study was also evaluated in a simultaneous randomized therapeutic HIV vaccine trial (7). The rationale for that was the high number of epitopes shared between these two vaccines (large stretch of CD4 and CTL epitopes from Gag/Pol/Nef in addition to Env gp160, Rev, and Tat encoded by the DNA GTU-MultiHIV vaccine). However, despite a large breadth of polyfunctional $CD4^+$ and $CD8^+$ T cell responses against epitopes contained in the vaccines, we noted a lack of $CD8^+$ T cells against Gag, a highly desirable target for therapeutic vaccines associated with responses controlling HIV, which might be one of the reasons for the lack of control of HIV replication (7).

Recent studies using whole blood or cellular transcriptional analyses identified B cell signatures and innate immunity pathways associated with protection in SIV/simian HIV nonhuman primate challenge studies or with immunogenicity in various prophylactic HIV vaccine platforms (54, 55). The present study has focused on investigating the mechanisms underlying vaccine immunogenicity, not vaccine-induced protection. We identified a gene signature of MVA HIV-B administration, with increased abundance 2 wk after the first administration, of genes belonging to the cell cycle regulation. These included CDC20, CDCA5, CDCA7, p53, and TCR and TLR pathways.

Table II. Gene expression changes after the first MVA HIV-B injection as prime or boost across three different heterologous prime-boost strategies: list of 21 significant probes with fold change >1.5

Probe ID	Gene Symbol	Fold Change	Average Expression
ILMN_1663390	CDC20	2.34	6.42
ILMN_2285996	PCLAF	2.13	5.64
ILMN_1806040	TYMS	2.00	6.28
ILMN_1737184	CDCA7	1.93	7.20
ILMN_1801939	CCNB2	1.88	5.58
ILMN_1686097	TOP2A	1.88	5.54
ILMN_1809590	GINS2	1.85	5.49
ILMN_1651237	CDT1	1.79	4.96
ILMN_1683450	CDCA5	1.71	5.49
ILMN_1737205	MCM4	1.67	6.28
ILMN_1786065	UHRF1	1.65	6.10
ILMN_1670238	CDC45	1.65	5.03
ILMN_2368718	CENPM	1.63	5.88
ILMN_2301083	UBE2C	1.62	5.82
ILMN_1678238	ZNF683	1.62	7.80
ILMN_1747016	CEP55	1.56	5.09
ILMN_1726720	NUSAP1	1.54	6.33
ILMN_1786125	CCNA2	1.53	5.36
ILMN_1749829	DLGAP5	1.53	4.95
ILMN_1806037	TK1	1.53	5.31
ILMN_3239771	DLGAP5	1.51	4.84

Time course gene expression analyses using mixed effect regression models with a random intercept and random slopes, and a variance component score test for significance testing at the probe level. All probes shown had a highly significant FDR-adjusted *p* value (FDR-adjusted *p* value = 0.000 for all 21 probes; highly significant *p* values take the value of zero due to the permutation test performed). The fold change indicates the absolute fold change between 2 wk after the first MVA HIV-B injection and the time point prior to injection. The average expression refers to the probe at the time point prior to the first MVA HIV-B injection. Results are sorted by fold change.

One striking observation in our study was the absence of variation in gene expression following a second administration of MVA HIV-B. One explanation could be the late time line of analysis (2 wk after each injection).

Of interest, compared with the literature, we were able to confirm in our clinical trial with human in vivo gene expression data 2 wk after the first MVA HIV-B administration the enrichment of two gene expression clusters of dendritic cells that have previously been identified in an in vitro experiment with the same vaccine (32).

In clinical vaccine trials in humans, at early sampling time points postvaccination, a gene expression signature of innate responses was reported at day 1 following MVA85A, a tuberculosis vaccine, in healthy infants, which was associated with vaccine-specific T cell responses (56). Innate gene expression signatures were also identified for other viral vector vaccines at an early time point (day 1 postvaccination) (57, 58). At the late sampling time point (2 wk after injections), we have previously reported that inflammatory pathways related to TLR signaling pathways, in response to a therapeutic vaccination using a dendritic cell–based HIV vaccine, were associated with a poorer immune response to vaccination and poorer viral control after treatment interruption (59).

Further efforts are required to perform early-phase clinical testing of various vaccine regimens in humans in a standardized manner to accelerate the decision to move best-in-class strategies into more advanced clinical development. Such shortened processes would be valuable to accelerate HIV vaccine development (60). We report in the present study an optimized phase I/II trial that allowed an unbiased evaluation of four heterologous prime-boost HIV vaccine strategies in parallel groups. Although we show that three out of four of these prime-boost vaccine strategies led to $CD4^+$ and $CD8^+$ T cell responses, those responses were primarily due to the MVA HIV-B component used in these strategies, which also drove changes in whole-blood gene expression. In total, we demonstrated that the ANRS MVA HIV-B was a safe and immunogenic T cell vaccine when given as either prime or boost in heterologous combinations. These results provided insights to pursue the development of this vector in an HIV therapeutic vaccine clinical trial (EHVA-T02, NCT04120415).

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Disclosures

The authors have no financial conflicts of interest.

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