A randomized placebo-controlled efficacy study of a prime boost therapeutic vaccination strategy in HIV-1 infected individuals: VRI02 ANRS 149 LIGHT Phase II trial

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ABSTRACT

In this placebo-controlled phase II randomized clinical trial, 103 HIV-1 infected patients under c-ART (combined antiretroviral treatment) were randomized 2:1 to receive 3 doses of DNA GTU-MultiHIV B (coding for Rev, Nef, Tat, Gag and gp160) at Week (W)0, W4 and W12 followed by 2 doses of LIPO-5 vaccine containing long peptides from Gag, Pol and Nef at W20 and W24 or placebos. Analytical treatment interruption (ATI) was performed between W36 to W48.

At W28, vaccinees experienced an increase in functional $CD4^+$ T cell responses measured ($P<0.001$ for each cytokine compared to W0) predominantly against Gag and Pol/Env and an increase in HIV-specific $CD8^+$ T cells producing IL-2 and TNF-α ($P=0.001$ and 0.013, respectively), predominantly against Pol/Env and Nef. However, analysis of T cell subsets by mass cytometry in a subpopulation showed an increase of W28/W0 ratio for memory $CD8^+$ T cells co-expressing exhaustion and senescence markers such as PD-1/TIGIT ($P=0.004$) and CD27/CD57 ($P=0.044$) in vaccinees compared to placebo. During ATI, all patients experienced viral rebound with a maximum observed HIV RNA level at W42 (median: 4.63 $\log_{10}$ cp/ml; IQR 4.00-5.09) without any difference between arms. No patient resumed c-ART for CD4 cell count drop. Globally, the vaccine strategy was safe. However, a secondary HIV transmission during ATI was observed.

These data show that the prime-boost combination of DNA and LIPO-5 vaccines elicited broad and polyfunctional T cells. The contrast between the quality of immune responses and the lack of potent viral control underscores the need of combined immunomodulatory strategies.

IMPORTANCE

In this placebo-controlled phase II randomized clinical trial, we evaluated the safety and immunogenicity of a therapeutic prime-boost vaccine strategy using a recombinant DNA vaccine (GTU®-MultiHIV B clade) followed by a boost vaccination by a lipopeptide vaccine (HIV-LIPO-5) in HIV-infected patients while on combined antiretroviral therapy. We show that this prime-boost strategy is well tolerated, consistently with previous studies in HIV-1 infected individuals and healthy volunteers who received each vaccine component individually. Compared to placebo group, vaccines elicited strong and polyfunctional HIV-specific $CD4^+$ and $CD8^+$ T cell responses. However, these immune responses presenting some qualitative defects were not able to control viremia following antiretroviral treatment interruption as no difference in HIV viral rebound was observed in vaccine and placebo groups. Several lessons were learned from these results pointing out the urgent need to combine the vaccine strategies with other immune-based interventions.

INTRODUCTION

Despite the beneficial effects of cART on HIV morbidity and mortality, these drugs do not eradicate the latent HIV reservoir resulting in a constant rebound in viremia after stopping cART (1). Several strategies are under development to clear latently infected cells, which contain integrated HIV DNA, capable to survive indefinitely in patients despite long-term cART. The concept behind these strategies, which needs to be proven, is that activation of these cells using latency reversing agents for instance, may lead to HIV reactivation, expression of HIV proteins and elimination of these cells by the immune system (2). Until now, clinical outcomes using this strategy have been disappointing. One possible obstacle is that the killing of cells harboring HIV requires robust and efficient T-cell responses making therapeutic vaccination central in strategies aiming at reducing the latent HIV reservoir and at achieving a functional cure (3).

In the last 25 years, several vaccine strategies to restore and improve HIV-specific functional immune responses have been developed, with varying results in terms of immunogenicity or HIV control when experimental designs comprised a period of ART interruption. In some trials, a partial effect on viral rebound was observed (4). Although promising, firm conclusions on the efficacy of these strategies are difficult to draw when they are based on non-controlled studies (4).

In the present study, we sought to address some of these issues by designing a randomized, placebo-controlled therapeutic vaccination trial combining two different HIV vaccines, GTU®-MultiHIV B clade and long HIV lipopeptide

sequences (HIV LIPO-5 vaccine) in a prime-boost regimen. These two vaccines share homologous HIV sequences and strong CTL epitopes. The GTU®-MultiHIV B encodes for a MultiHIV antigen (Rev, Nef, Tat, Gag p17/p24 proteins and an epitope stretch of previously identified CTL epitope-rich regions encoded by pol and env of a subtype B HIV-1 isolate Han-2) and lipopeptides are composed of 5 synthetic peptides (Nef 66-97, Nef 116-145, Gag 17-35, Gag 253-284, Pol 325-355 also from a clade B strain) to which lipid chains are covalently bound. GTU®-MultiHIV B has been evaluated in HIV untreated patients where it led to HIV-specific sustained $CD4^+$ and $CD8^+$ T cell response as well as a significant decline of plasma HIV viral load (5). More recent results combining transcutaneous (TC) and intramuscular (IM) injection of GTU®-MultiHIV B showed a lack of improvement of immune responses in HIV treated patients and concluded for the need of a combinatory approach (6). LIPO-5 has been evaluated in healthy adults where it led to a sustained HIV-specific $CD4^+$ and $CD8^+$ T-cell responses (7, 8).

Correlates of protection/control/cure are supposed to be different from HIV prophylactic interventions and especially T cell responses are supposed to play a key role in the clearance of infected cells. Successful approaches in SIV such as the CMV-based vaccine have given new bases on the key role of $CD8^+$ T cell response (9). There is a consensus in the field to propose ATI for evaluation of the virological efficiency of an immune intervention on the HIV reservoir since the ultimate objective of any intervention in HIV treated patients is to maintain a low viral replication after cART withdrawal (10). The efficacy endpoints of our study

included T-cell immunogenicity and viral kinetics following a 12-week period of antiretroviral treatment interruption (ATI).

RESULTS

Study participants

One hundred and thirty-three HIV-infected individuals were screened, and 103 were enrolled and randomized in 18 centers in France between September 2013 and May 2015 (Figure 1B). Five participants withdrew consent before receiving any intervention, 98 received at least one injection of placebo (n=35) or vaccine strategy (n=63) and were included in the mITT analysis of the study. Baseline characteristics of participants are reported in Table 1. The two study arms were balanced at baseline. Nine participants (2 and 7 in placebo and vaccine groups, respectively) withdrew from further follow up after week 0, the majority due to consent withdrawal.

Global overview of vaccine immunogenicity

Evaluation of vaccine-elicited T cell responses measured by flow cytometry-based intracellular cytokine staining (ICS) for antigen-specific IFN-γ and/or IL-2 and/or TNF-α was performed at baseline and week 28 on ninety-two participants who received the complete schedule of vaccination/placebo until week 28 in a per protocol analysis. Total $CD4^+$ T cell responses to several HIV peptides pools showed no difference between groups at entry. We found a significant increase of $CD4^+$ T cells producing cytokines to HIV peptide pools in the vaccine group at week 28 compared to baseline ($P<0.001$) while no difference was observed in the placebo group (Figure 2A). Detailed analysis for each cytokine and each HIV peptide pool showed a significant increase in IFN-γ, IL-2 and TNF-α production after Gag and Pol/Env stimulation, but not Nef, in the vaccine group (Figure 2C)

as well as increases of $CD4^+$ T cells producing 1, 2 or 3 cytokines at W28 ($P<0.001$ for all comparisons to baseline) (Figure 2D), specially against Gag and Pol/Env pools ($P<0.001$ for each comparison) (Figure 2F). While $CD8^+$ T cells producing cytokines against HIV peptides did not change in the placebo group, we found an increase of total cytokine in the vaccine group at week 28 compared to baseline (Figure 2B), and detailed analysis showed that these responses were directed against Pol/Env and Nef peptides, but not Gag (Figure 2C). The frequency of polyfunctional $CD8^+$ T cells (producing at least 2 cytokines), but not of monofunctional $CD3^+CD8^+$ T cells (producing only 1 cytokine), increases significantly in the vaccine group compared to W0 ($P=0.04$ and 0.025 for production of 2 and 3 cytokines, respectively) (Figure 2E), especially after Pol/Env or Nef stimulation (Figure 2F).

To extend the analysis of immune cells, an ancillary analysis of T cell phenotypic profile was performed in 28 patients (12 placebo and 16 vaccinees) with mass cytometry allowing the detection of 40 cell surface markers. Figure 3 depicts the W28/W0 ratio of gated positive populations for each marker in vaccinees compared to placebo. Significant changes of $CD8^+$ memory T cell subsets were observed in the vaccine group after vaccination with a higher frequency of memory $CD8^+$ T cells coexpressing PD-1 and TIGIT (Figure 3A), and coexpressing CD27 and CD57 (Figure 3B). Changes in the population of $CD8^+$ T cell exhibiting markers of activation were also observed as memory HLA DR^+ $CD38^-$ $CD8^+$ T cells were increased in the vaccine group (Figure 3C) without any modification of the memory $CD4^+$ T cells (Figure 3D). No change was observed in the different $CD4^+$ T cell subsets, including Treg or $CD32a^+$ expressed on

175 $CD4^+$ T cell HIV reservoir (not shown).

176

177 **Analytical Treatment Interruption**

178 ATI was proposed in both arms to individuals with plasma HIV RNA < 50
179 copies/ml at week 36. Eighty-nine participants (n=32 (91%) and 57 (90%) in
180 placebo and vaccine groups, respectively) started ATI according to study
181 protocol and were followed until week 48, the final study end point. Sixty-five
182 participants (n=23 (66%) and 42 (67%) in placebo and vaccine groups,
183 respectively) resumed ART according to the study protocol at week 48. Eighteen
184 participants (n=7 (20%) and 11 (17%) in placebo and vaccine groups,
185 respectively) resumed ART before week 48 for participants or doctors' decisions.
186 Two participants from each group resumed ART after week 48, and two
187 participants from the vaccine group did not resume ART at the end of the follow
188 up after week 48. Figure 4A shows longitudinal evolution of HIV plasma viral
189 loads (VL) in the two study arms during the ATI period. The maximum level of
190 viral load was observed at week 42 in both groups. mITT analysis did not show
191 any significant differences between groups in terms of maximum observed (peak)
192 viral load: median peak VL (Q1;Q3) between weeks 36-48 were 5.26 (4.58;7)
193 and 5.15 (4.73;7) in placebo and vaccine groups, respectively (P=0.9). The
194 frequency of participants with VL below 10 000 copies/ml at week 48, defined as
195 the virological success, was 50% and 44% in placebo and vaccine groups,
196 respectively. In total, seventy-one patients met this predefined success criterion
197 of the strategy without any significant difference between groups: 25 and 46 in
198 placebo and vaccine groups, respectively (Table 2).

199 The kinetics of the peak of VL looks slightly different between groups having
200 experienced ATI. At week 40, the maximum peak of VL was observed in 44%
201 and 28% of participants in the placebo and vaccine groups, respectively
202 (P=0.27). They were 8% and 20% at week 44, respectively (Table 2). At the end
203 of the ATI phase (week 48), two participants from the vaccine group did not
204 resume ART because of plasma VL below 50 copies/ml. These participants had
205 an initial VL rebound at week 42 and 44, and then exhibited a spontaneous
206 suppression of viremia which remained undetectable without ART at the end of
207 the study. Among the 91 participants restarting cART, 75 (31 and 44 in placebo
208 and vaccine group, respectively) participants suppressed viremia (<50 copies/ml)
209 at W74.
210 Figure 4B shows patterns of $CD4^+$ T cell changes in participants during the ATI
211 period. The evolution was similar in both groups of participants with a nadir
212 (median, IQR) at week 44 of 657 cells/mm^3 (556-832) and 661 (584-930) in
213 placebo and vaccine groups, respectively. $CD4^+$ T cell counts remain similar in
214 both groups at the end of the study and after resuming ART at week 48.
215
216 **Relationship between polyfunctionality of HIV-specific T cell responses and**
217 **viral parameters following ATI**
218 A principal component analysis (PCA) was conducted to illustrate the
219 interrelationships between vaccine-induced T cell responses measured by ICS
220 before ATI (polyf CD4, polyf CD8) and viral parameters during ATI.
221 Figure 5A is a projection of variables on the first two axes. The first principal
222 component (x-axis) represented 59% of the variability while the second principal

component represented 21%. All immunological variables were on the right side of the figure, illustrating their trend to be positively correlated. The y-axis allowed to differentiate the $CD8^+$ T cell responses (top) and the $CD4^+$ T cell responses (bottom). Results showed that maximal viral load, viral load slope and viral load AUC were projected at the opposite direction of the immune markers, indicating a trend towards negative correlations between the magnitude of viral load after ATI and T cell responses before ATI. In Fig. 5B, the representation of the patients on the PCA illustrates the poor immunological status of some patients (left side) and the $CD4^+$ and $CD8^+$ T cell-oriented response of the others (middle right). Vaccinated participants seemed to be slightly more numerous on the right part of the plan (as shown by the distribution blue curve on the top) corresponding to good responders, while those who were on the left part exhibited poorer immunological responses (being vaccinated or not) with a higher maximum viral load.

Safety

Ninety-eight individuals received at least one injection, 93 received all injections. The majority of participants (96%) experienced at least one Adverse Events (AE) being transient (median duration 15 days, IQR 3-62). As shown in Table 3, most of the AE were grade 1 or 2 and there were no marked differences between arms. Among 15 Serious Adverse Events (SAE) (Table 4), one was possibly related to the GTU®-Multi-HIV B vaccine (arthritis) and one to the research: secondary HIV transmission during the ATI period confirmed by phylogenetic analysis of the HIV in the placebo arm (11). Primary infection-like symptoms,

usually mild, were observed in 23% of the individuals after ATI. There was no resumption of ART due to CD4 cell count drop during ATI.

DISCUSSION

In this study, we show that a therapeutic immunization strategy combining a DNA prime followed by a boost with long HIV lipopeptides is well tolerated in chronically HIV-1 infected individuals treated with cART. These safety data are consistent with previous studies in HIV-1 infected individuals and healthy volunteers who received each vaccine component individually (8, 12, 13) .

This study comprised two phases, a vaccination period followed by an ATI phase of 12 weeks to evaluate both the immunogenicity and virologic efficacy of the vaccine strategy.

At the end of the vaccination period, the immunogenicity of the vaccine strategy was clearly demonstrated. Vaccinees exhibited significant changes in the frequency and the functionality of HIV-specific T cell responses. However, these changes in the immune status of individuals did not translate into any differences in the kinetics and magnitude of viral rebound following ATI. Consistently, we found that the vaccine strategy did not impact significantly the levels of cellular HIV-DNA measured before ATI (14). Nevertheless, integrative analysis of virological and immunological parameters showed a trend toward an association between good vaccine responders and a lower viral load after ATI, while individuals with poorer immunological responses (being vaccinated or not) exhibited a higher maximum viral load.

These findings might have important implications in the design and evaluation of future studies testing immunological interventions aimed at sustainably control viral replication without cART.

The rationale to combine a DNA GTU prime and HIV long lipopeptides was based on previous results obtained with each individual vaccine component. Administration of DNA GTU in cART naïve individuals resulted in a modest, but significant, decrease of plasma HIV viral load (up to 0.5 $\log_{10}$ copies/ml) in a large therapeutic study performed in South African individuals (5). Previous therapeutic vaccine studies centered around HIV lipopeptides provided also encouraging results (7, 8). Combination of ALVAC/HIV lipopeptide and IL-2 preceding ATI in chronically HIV-1 infected patients resulted in a greater chance to maintain a viral load during a 24 weeks ATI period (HIV RNA below 10 000 copies/ml as predefined in the present study) compared to individuals from a control arm (12, 15). In a recent non randomized vaccine study, we showed that vaccination with *ex vivo* generated Dendritic Cells (DC) loaded with HIV-lipopeptides (the Dalia trial) elicited strong $CD4^+$ and $CD8^+$ T cell responses associated with a control of viral replication following ATI in chronically HIV-1 infected individuals (16). In these two previous studies, we found a correlation between vaccine elicited responses and the magnitude of viral replication or the frequency of individuals maintaining plasma HIV viral load below a predefined threshold following ATI (12, 15–18).

295 Here, the combination of these two vaccine components in a prime boost
296 strategy was also supported by the sharing of several HIV T-cell epitopes in
297 common, raising the hypothesis of a stronger induction of HIV-specific T cell
298 responses. Indeed, immunogenicity analysis showed a significant expansion of
299 functional T-cell responses (producing at least two cytokines) against HIV-1 Gag,
300 Env and Pol antigens for $CD4^+$ T cells while $CD8^+$ T cells were directed against
301 Env, Pol, Nef but not Gag antigens. Despite this broad repertoire, these
302 responses did not impact significantly HIV replication throughout the 12-weeks of
303 ATI. The failure to show an association between vaccine immunological efficacy
304 and the kinetics of viral rebound raises several questions about the repertoire,
305 functionality of these responses and the immunological context following
306 vaccination. In depth analysis and epitope mapping of T-cell responses elicited
307 by the DC-based vaccine delivering HIV lipopeptides revealed an inverse
308 correlation between the functionality of $CD4^+$ T cell responses (production of IL-2
309 and IL-13), the repertoire of these responses directed against HIV Gag, Nef and
310 Pol dominant epitopes, and the magnitude of viral rebound (17). These results
311 are supported by several previous studies showing that robust HIV-1-specific T
312 cell responses are associated with a better control of infection in LTNP (19). In
313 the present study we did not investigate the precise repertoire of $CD4^+$ T cell
314 responses against individuals HIV epitopes. Whether the lack of antiviral effect of
315 HIV-specific $CD4^+$ T cell responses to vaccine regimen containing HIV
316 lipopeptides delivered through IM route, as compared to DC delivery, could be
317 explained by a difference in the immune profile (cytokine pattern) or repertoire of
318 vaccine elicited $CD4^+$ T-cell responses warrants further analyses.

319

320 We also show that the vaccine regimen elicited expansion of memory $CD8^+$ T cell
321 responses. Surprisingly, responses against Gag epitopes contained in the DNA
322 GTU and Lipopeptide sequences were not amplified. Several teams, including
323 our group, have shown the importance of $CD8^+$ T cell responses to Gag in the
324 control of HIV (17). Our results, from a subgroup of individuals, show also
325 changes in the population of $CD8^+$ T cell exhibiting markers of activation
326 (increase of memory HLA DR^+ $CD38^-$ $CD8^+$ T cells in the vaccine group) and
327 more importantly markers of exhaustion (TIGIT and PD-1) and senescence
328 (CD57), which might indicate the low capacity of these cells to control viral
329 replication. These inhibitory immune receptors have been previously shown to
330 regulate antiviral and antitumor $CD8^+$ T cell effector function in mice model of
331 LCMV and in human with advanced melanoma (20–22). It has been shown that
332 TIGIT and PD-1 blockade additively increased proliferation, cytokine production,
333 and degranulation of tumor antigen-specific $CD8^+$ T cells. One limitation of this
334 observation is that we did not look at the expression of these markers on HIV-
335 specific $CD8^+$ T cells. However, as already described in cancer patients, we
336 cannot rule out that these specific $CD8^+$ T cells would exhibit a low killing
337 capacity of HIV infected cells (20). Regarding the design of future studies, these
338 results underscore the need to include functional killing assays in the evaluation
339 of the efficacy of vaccine trials (23).

340

341 One intriguing question, beyond the results of this trial, is why despite the
342 capability of eliciting strong immune responses, several candidate vaccines

343 tested showed disappointing results and failed to control HIV replication in cART-
344 free individuals. We, and others, have already raised the hypothesis that the
345 balance between inflammatory responses and activation of effector T cells
346 seems crucial in this setting (24). The deleterious association of persistent
347 inflammation signature after vaccination with the immune response to vaccine
348 has been reported for several vaccine platforms (25, 26), including HIV (24).
349 Recently, integrative analysis of a large set of arrays (T-cell responses, cytokine
350 production, blood transcriptomic changes) evaluating immune responses in
351 individuals receiving DC/HIV lipopeptide vaccine showed that inflammatory
352 pathways related to Toll-Like Receptor signaling were associated with a poorer
353 immune response to vaccination and poorer viral control after ATI (24). The
354 similar involvement and impact of these pathways in responses to other vaccines
355 indicates a potential broad mechanism driving the immune response to vaccine.
356 Likely these data underscore the need to carefully investigate, besides the profile
357 of effector specific T cells, the kinetics of inflammatory responses in future
358 vaccine studies. Furthermore, these results point out the need to develop further
359 strategies combining vaccines with adjuvants and/or immunomodulators (3).
360
361 The lack of immune correlates, or robust markers, predicting virologic control
362 implies that a period of antiretroviral treatment interruption remains necessary to
363 assess the efficacy of immune interventions in HIV-infected patients. Our study
364 comprised a 12-weeks ATI period and an arbitrary threshold of plasma viral load
365 defining the success of the strategy (i.e: frequency of individuals maintaining
366 plasma viral load below 10 000 copies/ml). One month following cART

interruption, the maximum peak of plasma viral load concerned a higher percentage of placebo as compared to vaccinees (44% and 28% of participants, respectively). At the end of the ATI phase (week 48), two participants from the vaccine groups maintained a suppressed viral load below 50 copies/ml and remained without cART at week 74. Interestingly these participants had an initial VL rebound at week 42 and 44 which makes unlikely that these two subjects were elite HIV controllers. However, we were unable to demonstrate the efficacy of vaccine regimen in an intent-to-treat analysis and according to the predefined criteria of success. This underscores the added value of the comparison to a well-controlled placebo group (4) to limit the risk of misinterpreting results. The decision to propose ATI to individuals receiving placebo should be carefully balanced by the risk to miss the demonstration of efficacy or to erroneously conclude on existing efficacy of an immune intervention. Thus, the large heterogeneity of previous immunotherapeutic trials in terms of ATI duration, presence of a control group, threshold criteria for resuming cART, timeline of virologic evaluation might hinder the capacity to identify promising strategies. For example, the use of a conservative criterion for resuming cART, such as plasma viremia above 1 000-2 000 copies/ml, risks missing important positive effects of immune interventions on viral control (27). Likely, the recent consensus report on recommendations to optimize ATI strategies and to mitigate the risks for participants will help to better design future studies.

In order to minimize the risks for participants undergoing ATI, in our trial we used strict safety criteria for resuming cART before the end of the 12-week period of

ATI, such as a confirmed >30% decline in $CD4^+$ T cell count, an absolute $CD4^+$ T cell count <350 cells/mm^3, or the development of acute retroviral syndrome. Globally the strategy was well tolerated and no individuals reached these safety criteria for resuming cART.

However, despite strong measures of counseling, ATI was associated with a secondary transmission to a sex partner of one participant from our study (11). This observation led our group to propose PrEP in our future HIV cure trial in France (28) but also at the European level (EHVA T02 trial NCT04120415; (29)). Although PrEP may mitigate the risk of secondary transmission, this strategy should be associated with strong counseling and additional measures of prevention because of the lack of clear data on the efficacy of PrEP against viral rebound to high levels of viremia following ATI. It would be also essential to closely monitor plasma viral load in participants during the ATI period and to adapt PrEP drugs to the resistant profile to the participant's virus.

In conclusion, the prime boost regimen tested in this study was designed to maximize the immune response and to evaluate its virologic efficacy in a well-controlled design trial including a long-term ATI period. This study adds to the list of previous therapeutic vaccine trials showing that despite eliciting strong immune responses, no association to a long-term control of viremia was demonstrated. However, several lessons were learned from these results pointing out the urgent need to combine these vaccine strategies with other immune-based interventions.

MATERIALS AND METHODS

Study design and participants

The VRI02 ANRS 149 LIGHT trial is a phase II randomized, placebo-controlled, double-blinded, multicenter trial evaluating the safety and immunogenicity of a prime-boost vaccine strategy using a recombinant DNA prime vaccine (GTU®-MultiHIV B clade) followed by a boost vaccination by a lipopeptide vaccine (HIV-LIPO-5) in HIV-infected patients while on cART. Eligible patients were asymptomatic HIV-1-infected adults with $CD4^+$ T-cell counts >600 cells/µL, plasma HIV RNA <50 copies/mL at screening and within the previous 6 months while on cART and were recruited in 18 hospitals in France. All study participants provided written informed consent before participation. The protocol was approved by the ethics committee of Ile de France 5 (Paris-Saint-Antoine) and authorized by the French regulatory authority (ANSM). The study is registered at www.ClinicalTrials.gov (NCT01492985) and EudraCT: 2009-018198-30.

Randomization and masking

Participants were randomized in a 1:2 ratio to receive either placebo or active vaccine. Randomization was done centrally one week before the first vaccination via the electronic case report software (Ennov clinical® software), on the basis of a randomization list generated by the unblinded statistician (CMG-EC, Inserm U1219, Bordeaux). Site staff and participants were both masked to the treatment assignment.

Procedures

DNA GTU MultiHIV and HIV LIPO-5 have been described elsewhere (5–7). Briefly, GTU®-MultiHIV B clade, developed by FIT Biotech, encodes for a MultiHIV antigen (synthetic fusion protein built up by full-lenght polypeptides of Rev, Nef, Tat, p17 and p24 with more than 20 Th and CTL epitopes of protease, reverse transcriptase (RT) and envgp160 regions of the HAN2 HIV-1 B clade. HIV-LIPO-5 vaccine consisted in 5 long HIV peptides from (Nef 66-97; Nef 116-145; Gag 17-35; Gag 253-284 and Pol 325-355) to which lipid tail are covalently bound. These lipopeptides which cover HIV epitopes binding to > 90% of HLA molecules, permit presentation of $CD4^+$ and $CD8^+$ T cell epitopes as well as generation of humoral immunity (17).

The DNA GTU MultiHIV at a dose of 1 mg or placebo priming vaccinations were administered IM using a biojector at study weeks 0, 4 and 12. HIV-LIPO-5 boosts, at a dose of 2.5 mg (0.5 mg of each lipopeptide) or placebo were given at weeks 20 and 24 (Figure 1A). For immunological analysis, PBMC samples were collected at entry (W0), four weeks following the last DNA GTU prime (W16) and the last LIPO-5 boost (W28), W48 (final end point) and W74. A cART interruption between W36 and W48 was proposed to individuals who had HIV-1 RNA <50 copies/mL and $CD4^+$ T-cell counts >600 cells/µL. Clinical, immunological ($CD4^+$ and $CD8^+$ T cell counts) and virological (HIV viral load) follow-up was performed every 15 days for 2 months during ATI, then monthly. cART has to be resumed at W48 but could be resumed at any time according to the following criteria: (i) if the patients or their doctors wished so; (ii) if $CD4^+$ T-cell count was <350 cells/µL at two consecutive measurements 2-weeks apart; and (iii) in the case of occurrence

of an opportunistic infection or a serious non-AIDS defining event. Patients were followed until W74 for final safety evaluation after resuming cART.

Intracellular cytokine staining (ICS) assay

Cell functionality was assessed by ICS, with Boolean gating to examine vaccine-induced HIV-1-specific $CD4^+$ and $CD8^+$ T cell responses after stimulation with 3 different HIV 15-mer peptide pools (1 pool Gag, 1 pool Pol/Env and 1 pool Nef peptides, JPT Peptide Technologies GmbH, Berlin, Germany). SEB-stimulated and unstimulated cells were used as positive and negative control, respectively. The flow cytometry panel included a viability marker, CD3, CD4 and CD8 to determine T-cell lineage, and IFN-γ, TNF-α, and IL-2 antibodies. Data were acquired on a LSRFortessa 4-laser (488, 640, 561 and 405 nm) cytometer (BD Biosciences) and analyzed using FlowJo software version 9.9.4 (Tree Star inc.).

Mass cytometry staining and analyses

In a subpopulation of patients from both groups selected among those presenting an ICS response, a mass Cytometry (CyTOF) analysis was performed at weeks 0 and 28. PBMC were thawed, rested and then stained using metal-conjugated antibodies according to the CyTOF manufacturer's instructions (Fluidigm, San Francisco, CA). Cell viability staining was performed using the Cell-ID™-103 Rh Intercallator at a final concentration of 1 µM that was incubated with PBMC for 15 minutes. PBMC from an individual donor were treated in parallel and multiplexed for staining and mass cytometry analysis to limit sample variation due to sample preparation and analysis. Multiplexed week 0 and week 28 PBMC were stained

for 20 minutes with either anti-CD45 89 Y or anti-CD45 194 Pt isotopes, respectively, and then washed with CSM buffer (PBS, 0.5% BSA, 0.02% sodium azide, all from Sigma) before combining the two samples. Pooled samples containing 2-4x10^6 cells were stained for 30 minutes using a cocktail of antibodies for cell surface markers in a total volume of 50 µl (Table 5). Cells were subsequently washed with CSM and PBS, fixed with 2.4% formaldehyde (Thermo Fisher) in PBS for 5 minutes and then resuspended in DNA-intercalation solution (PBS, 1 µM Ir-Intercalator, 1% formaldehyde, 0.3% saponin) before storage at 4°C until analysis. For CyTOF analysis, cells were washed 3 times with MilliQ water and resuspended at 0.5x10^6 cells/ml in 0.1% EQ™ Four Element Calibration Beads solution (Fluidigm). Samples were normalized for the EQ™ bead intensities using the matlab normalizer software to limit inter analysis staining intensities. Data were processed and analyzed with cytobank. Since W0 and W28 samples for a given donor were multiplexed and stained in parallel, the relative changes in marker intensities were determined using the W28/W0 ratio for the indicated gated positive populations.

Study endpoints

The primary endpoint was the maximum observed plasma HIV-1 RNA load (in log_{10} copies/mL) during the ATI period between W36 and W48. Participants not having interrupted cART at W36, or having resumed their treatment before W48, were imputed with the maximum plasma HIV-1 RNA load observed among all the participants during the ATI. The delay to the maximum plasma viral load was also described in participants having experienced the ATI between W36 and W48.

Clinical and virological secondary endpoints were as follows: the frequency of clinical and biological adverse events occurring during the trial; $CD4^+$ T cell counts at W40, W44, W48, W74; HIV-1 RNA loads at W40, W44, W48, W74; the virological success, assessed as the percentage of participants with plasma HIV-1 RNA load below 10 000 copies/mL at W48 considering virological failure for participants not having interrupted cART at W36; proportion of participants who reinitiated ARTs after W36; proportion of participants with $CD4^+$ T cell counts < 350/mm^3.

Secondary immunological end points were ICS based on the boolean and the marginal percentages of cells producing IFN-γ, IL-2 and TNF-α per T cell population ($CD3^+CD4^+$ and $CD3^+CD8^+$) after HIV stimulation (Gag, Pol/Env, Nef and total HIV) with background subtraction (negative values obtained after removing background were imputed to zero). The percentages of cells producing at least one cytokine among IFN-γ, IL-2 and TNF-α and polyfunctional cells (cells producing at least two or three cytokines) were also described.

Statistical analysis

The sample size calculation was based on the assumption that a reduction by at least 0.7 log_{10} copies/mL in plasma HIV-1 RNA level at the end of the ATI in the vaccinated group compared to the placebo group (standard deviation of the viral load at the end of the interruption estimated at 1.0 log_{10} copies/mL in the Window/ANRS 106 trial). With a two-sided type I error of 5% and a power of at least 90% (Wilcoxon-rank test), the targeted number of participants was 35 in the placebo group and 70 in the vaccine group.

All efficacy and safety analyses were carried out as modified intention to treat (mITT), in which participants who received no vaccine dose were excluded from the analysis. The immunological analyses were based on the per protocol population defined as exclusion from the analyses of participants with any discontinuation regarding either the vaccine therapy or the ATI. Quantitative and qualitative variables were respectively described by median and inter-quartile range and by frequency and proportion.

The primary endpoint expressed as the maximal plasma viral load during the ATI was compared between the placebo and the vaccine groups with the two-sided Wilcoxon-rank test. The immune ICS responses were compared between W0 and W28 in each arm using the Wilcoxon signed-rank test. A principal component analysis across ICS responses at W28 (log-transformed marginal % of positive cells for IFN-γ, IL-2, TNF-α, per T cell population, $CD4^+$ and $CD8^+$ respectively) was performed with a projection of highest viral load during ATI, viral load slope, time to rebound and viral load AUC as supplementary variable.

Statistical analyses were performed using SAS (version 9.3 or higher, SAS Institute, Cary, NC, USA) and R (version 3.6.0, The R Foundation for Statistical Computing, Vienna, Austria). Tests with a two-sided *P* value of <.05 were considered statistically significant.

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736 **Figure legends**

737

738 **Figure 1: Trial design**. (A) Schematics of study design. Blue arrows indicate
739 time of DNA GTU-MultiHIV B or placebo administrations. Red arrows indicate
740 time of HIV LIPO-5 or placebo administrations. ART, antiretroviral therapy; ATI,
741 analytical treatment interruption; (B) Consolidated Standards of Reporting Trials
742 (CONSORT) flow diagram for the trial. CONSORT diagram delineates the study
743 enrollment of 103 participants who underwent randomization to the placebo or
744 vaccine groups.

745

746 **Figure 2: Functional profile of $CD4^+$ and $CD8^+$ T cell responses.** Production
747 of IL-2, IFN-γ and TNF-α as measured by intracellular cytokine staining (ICS)
748 using multiparametric flow cytometry after cell stimulation before (W0) and after
749 vaccination (W28) in placebo (red) and therapeutic vaccine (blue) groups: (A)
750 HIV-specific $CD3^+CD4^+$ T cell frequency; (B) HIV-specific $CD3^+CD8^+$ T cell
751 frequency; (C) Heatmap of P values between W28 and W0 of $CD3^+CD4^+$ and
752 $CD3^+CD8^+$ marginal responses against Gag, Pol/Env, Nef and sum of HIV
753 peptides (Total HIV); (D) Frequency of HIV-specific $CD3^+CD4^+$ T cells producing
754 1, 2 or 3 cytokines in the vaccine group at W0 (light grey) and W28 (dark grey);
755 (E) Frequency of HIV-specific $CD3^+CD8^+$ T cells producing 1, 2 or 3 cytokines in
756 the vaccine group at W0 (light grey) and W28 (dark grey); (F) Heatmap of P
757 values between W28 and W0 of $CD3^+CD4^+$ and $CD3^+CD8^+$ polyfunctionality
758 responses against Gag, Pol/Env, Nef and total HIV peptides.

759

760 **Figure 3: CyTOF phenotyping.** Ratio of memory $CD8^+$ T cells at W28 compared
761 to W0 for several subsets according to PD-1 and TIGIT (A), CD27 and CD57 (B),
762 or HLA-DR and CD38 (C) in placebo (red) and therapeutic vaccine (blue) groups.
763 Ratio of memory $CD4^+$ T cells at W28 compared to W0 for HLA-DR and CD38
764 (D) in placebo (red) and therapeutic vaccine (blue) groups. P-values were
765 calculated using the Mann-Whitney test, where P=0.017 (*); P=0.0022 (**).
766
767 **Figure 4: Plasma HIV viral load and $CD4^+$ T cell count changes throughout**
768 **the study.** (A) Levels of plasma HIV RNA in the placebo (red) and therapeutic
769 vaccine (blue) groups before and after ATI (week 36-48); (B) $CD4^+$ T cell count
770 changes during the vaccination phase and following ATI in the placebo (red) and
771 therapeutic vaccine (blue) groups before and after ATI (week 36-48).
772
773 **Figure 5: Integrative analysis of immune response to vaccine.** Principal
774 component analysis of ICS responses at W28. Log-transformed marginal $CD4^+$
775 and $CD8^+$ T cell responses at W28 were included as active variables; virological
776 markers during ATI (highest viral load, viral load slope, viral load AUC, time to
777 rebound) were included as supplementary variables. (A) Projection of variables;
778 (B) Projection of individuals represented into placebo (red) and therapeutic
779 vaccine (blue) groups.
780
781

782 **Table 1: Baseline characteristics of study participants**

	Placebo (n = 35)	Vaccine (n = 63)	Total (n = 98)
Age in years*	44 (38 ; 49)	46 (36 ; 51)	45 (38 ; 51)
Male, n (%)	30 (86)	56 (89)	86 (88)
Time since first positive serology (in years)*	7 (5 ; 13)	8 (4 ; 14)	7 (4 ; 14)
Nadir $CD4^+$ ($/mm^3$)*	390 (335 ; 502)	389 (332 ; 480)	390 (334 ; 480)
$CD4^+$ count at baseline ($/mm^3$)*	844 (684 ; 1060)	840 (744 ; 1018)	842 (733 ; 1045)
RNA zenith (log_{10} cp/ml)*	5.1 (4.8 ; 5.6)	5.0 (4.4 ; 5.4)	5.0 (4.5 ; 5.4)
RNA at baseline (log_{10} cp/ml)*	1.6 (1.6 ; 1.6)	1.6 (1.6 ; 1.6)	1.6 (1.6 ; 1.6)

783 * *Median (Q1;Q3)*

784

785 **Table 2: Plasma HIV RNA values during ATI period**

	Placebo (n = 35)		**Vaccine (n = 63)**		**Total (n = 98)**		**P-value**
Maximum VL during ATI (log_{10} cp/ml)							
Mean (SD)	5.39	(1.40)	5.42	(1.17)	5.41	(1.25)	
Median (IQR)	5.26	(4.58 ; 7.00)	5.15	(4.73 ; 7.00)	5.16	(4.70 ; 7.00)	0.878
Range [min-max]		[1.60 – 7.00]		[1.60 - 7.00]		[1.60 - 7.00]	
ATI experience between W36 and W48, n (%)	25	(71)	46	(73)	71	(72)	
Time of maximum VL during ATI in participants having experienced ATI, n (%)							
W38	2	(8)	3	(7)	5	(7)	
W40	11	(44)	13	(28)	24	(34)	
W42	8	(32)	15	(33)	23	(32)	
W44	2	(8)	9	(20)	11	(15)	
W48	2	(8)	6	(13)	8	(11)	
Maximum VL during ATI (log_{10} cp/ml) in participants having experienced ATI	6.12		5.95		6.12		
Participants with VL below 10 000 cp/ml at W48*, n (%)							
No	17	(50)	34	(56)	51	(54)	
Yes	17	(50)	27	(44)	44	(46)	

** 4 participants did not resume ART at W36 and were considered in virological failure at W48*

786

787 **Table 3: Adverse events (AE) after W0**

	Placebo (n = 35)		Vaccine (n = 63)		Total (n = 98)	
Participants presenting at least one AE, n (%)	35	(100)	59	(94)	94	(96)
Participants presenting at least one biological AE, n (%)	5	(14)	9	(14)	14	(14)
Participants presenting at least one clinical AE, n (%)	35	(100)	59	(94)	94	(96)
AE by maximal grade, n (%)	220		405		625	
Grade 1 : Mild	84	(38)	217	(54)	301	(48)
Grade 2 : Moderate	123	(56)	170	(42)	293	(47)
Grade 3 : Severe	12	(5)	18	(4)	30	(5)
Grade 4 : Life threatening	1	(0)	-		1	(0)
SAE among all AEs, n (%)						
No	213	(97)	397	(98)	610	(98)
Yes	7	(3)	8	(2)	15	(2)
Median duration of AE (in days) (Q1;Q3)	24	(5;90)	12	(3;49)	15	(3;62)
Participants presenting at least one AE related to vaccine, n (%)	12	(34)	34	(54)	46	(47)
AE related to vaccine by maximal grade, n (%)	36		105		141	

Grade 1 : Mild	19	(53)	85	(81)	104	(74)
Grade 2 : Moderate	17	(47)	19	(18)	36	(26)
Grade 3 : Severe	-		1	(1)	1	(1)
Grade 4 : Life threatening	-		-		-	
SAE among AE related to vaccine , n (%)						
No	36	(100)	104	(99)	140	(99)
Yes	-		1	(1)	1	(1)

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791 **Table 4: Description of severe adverse events (SAE) after W0 by System Organ Class (SOC) and preferred term (PT)**

SOC	PT	Placebo (n = 7)	Vaccine (n = 8)	Total (n = 15)
Infections and infestations	Peritonitis, n (%)	1 (14.3)	.	1 (6.7)
	Bacterial rectitis, n (%)	.	1 (12.5)	1 (6.7)
	Transmitting the HIV infection, n (%)	1 (14.3)	.	1 (6.7)
Injury, poisoning and procedural complications	Accident on the public highway, n (%)	.	1 (12.5)	1 (6.7)
	Artery stenosis, n (%)	.	1 (12.5)	1 (6.7)
	Toxicity of various agents, n (%)	.	1 (12.5)	1 (6.7)
Musculoskeletal and connectives disorders	Arthralgia, n (%)	1 (14.3)	.	1 (6.7)
	Rheumatoïd arthritis, n (%)	.	1 (12.5)	1 (6.7)
Heart disorders	Congestive cardiomyopathy, n (%)	1 (14.3)	.	1 (6.7)
Reproductive system and breast disorders	Benign prostatic hypertrophy, n (%)	1 (14.3)	.	1 (6.7)
Nervous system disorders	Craniocerebral injuries + loss of consciousness, n (%)	.	1 (12.5)	1 (6.7)
Blood and lymphatic system disorders	Iron deficiency anemia, n (%)	1 (14.3)	.	1 (6.7)
Psychiatric disorders	Suicide, n (%)	1 (14.3)	.	1 (6.7)

Respiratory, thoracic and mediastinal disorders	Pulmonary disorder, n (%)	.	1 (12.5)	1 (6.7)
Metabolism and nutrition disorders	Diabetes, n (%)	.	1 (12.5)	1 (6.7)

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793
794

795 **Table 5 : Overview of the mass cytometry panel**

Marker	Isotope	Clone	Source	Vol (µl) per 50 µl
CD45	89 Y	HI30	Fluidigm	0.40
CD8	113 In	RPA-T8	Biolegend	0.50
CD4	115 In	RPA-T4	Biolegend	0.40
CCR6	141Pr	11A9	Fluidigm	0.50
CD19	142 Nd	HIB19	Fluidigm	0.80
ICOS	143 Nd	C398.4A	Biolegend	0.80
CD69	144 Nd	FN50	Fluidigm	0.50
CD31	145 Nd	WM59	Fluidigm	0.60
IgD	146 Nd	IA6-2	BD Biosciences	0.70
CD28	147 Sm	L293	BD Biosciences	0.30
CD57	148 Nd	G10F5	Biolegend	0.25
CCR4	149 Sm	205410	Fluidigm	0.75
OX40	150 Nd	ACT35	Fluidigm	120
CD103	151Eu	Ber-ACT8	Fluidigm	0.80
CD21	152 Sm	BL13	Fluidigm	0.50
TIGIT	153 Eu	MBSA43	Fluidigm	0.60
TLR2	154 Sm	TL2.1	Fluidigm	100
CD27	155 Gd	L128	Fluidigm	0.50
CD11c	156 Gd	3.9	Biolegend	0.60
CCR7	159 Tb	G043H7	Biolegend	0.30
CD14	160 Gd	M5E2	Fluidigm	0.97
CD1c	161Dy	L161	Biolegend	0.30
CD32a-APC	162 Dy	APC003	Fluidigm	3.5/10
CXCR3	163 Dy	G025H7	Fluidigm	0.60
CD45RO	165 Ho	UCHL1	Fluidigm	0.36
CD38	167 Er	HIT2	Fluidigm	0.30
CD40L	168 Er	24-31	Fluidigm	120
CD45RA	169 Tm	HI100	Fluidigm	0.80
CD3	170 Er	UCHT1	Fluidigm	0.40
LAG3	172 Yb	BMS	Biotechne	160
HLA-DR	173 Yb	L243	Fluidigm	0.30
PD1	174 Yb	EH12.2H7	Fluidigm	0.50
CXCR4	175 Lu	12G5	Fluidigm	0.35
CD127	176 Yb	A019D5	Fluidigm	0.70
CD45	194 Pt	HI30	Biolegend	0.50
CD16	209 Bi	3G8	Fluidigm	0.50

796

Figure 1

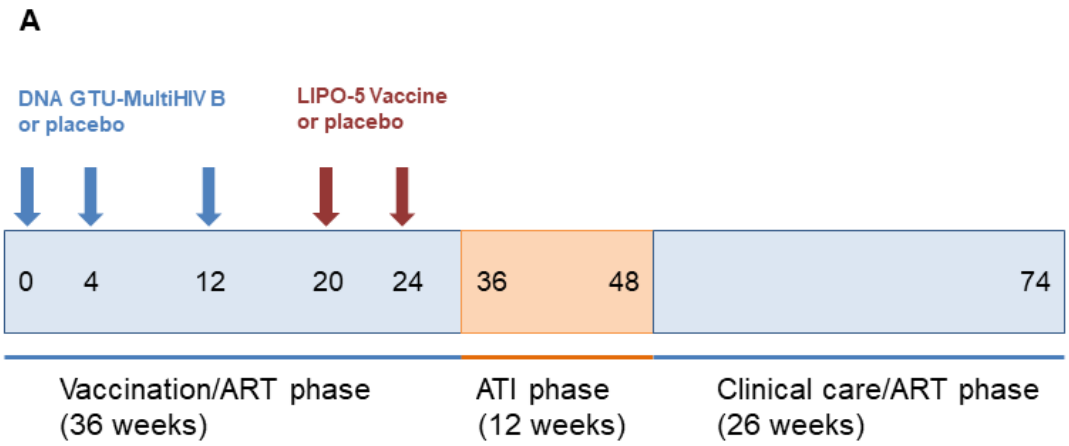

Figure 1

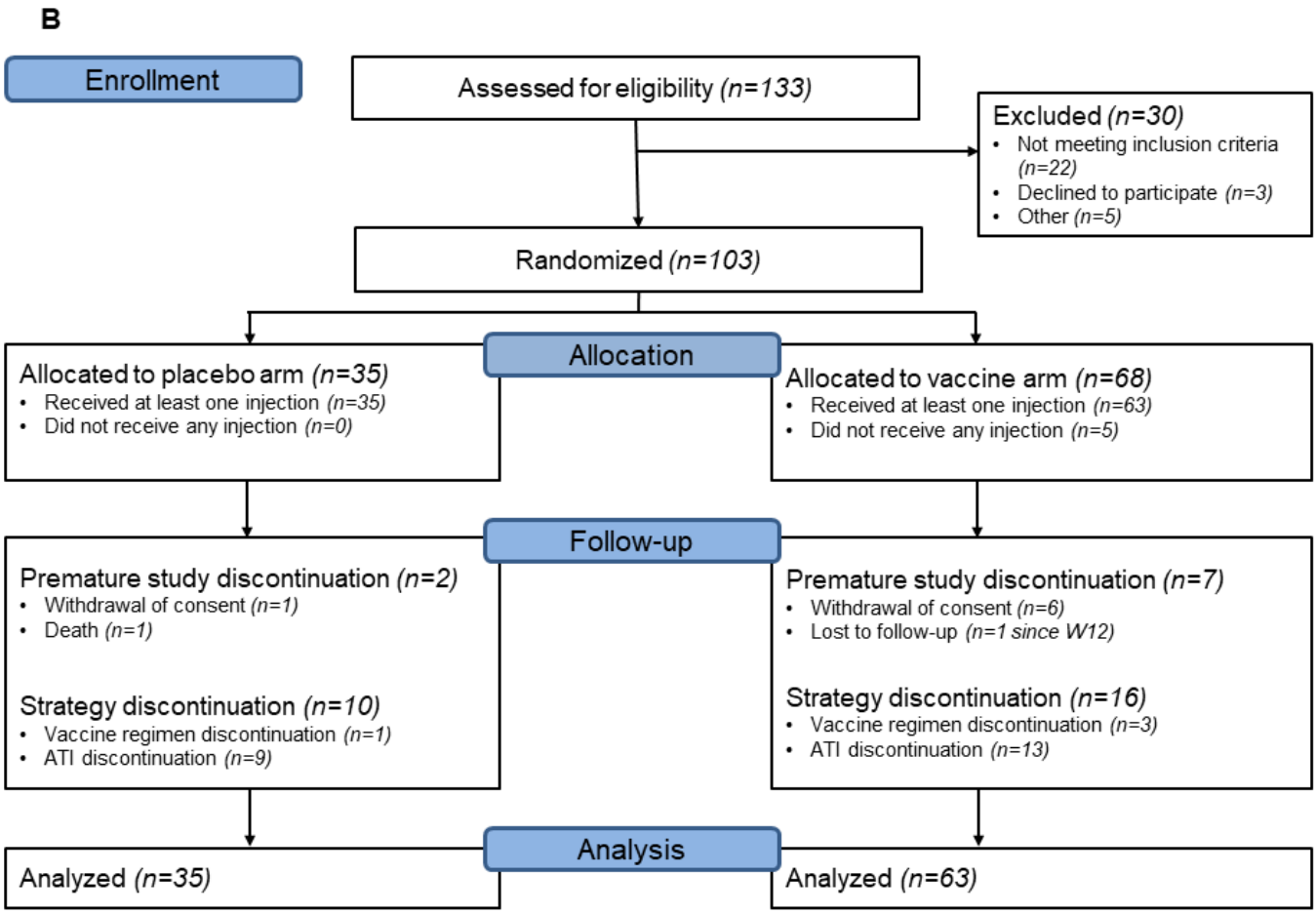

Figure 2

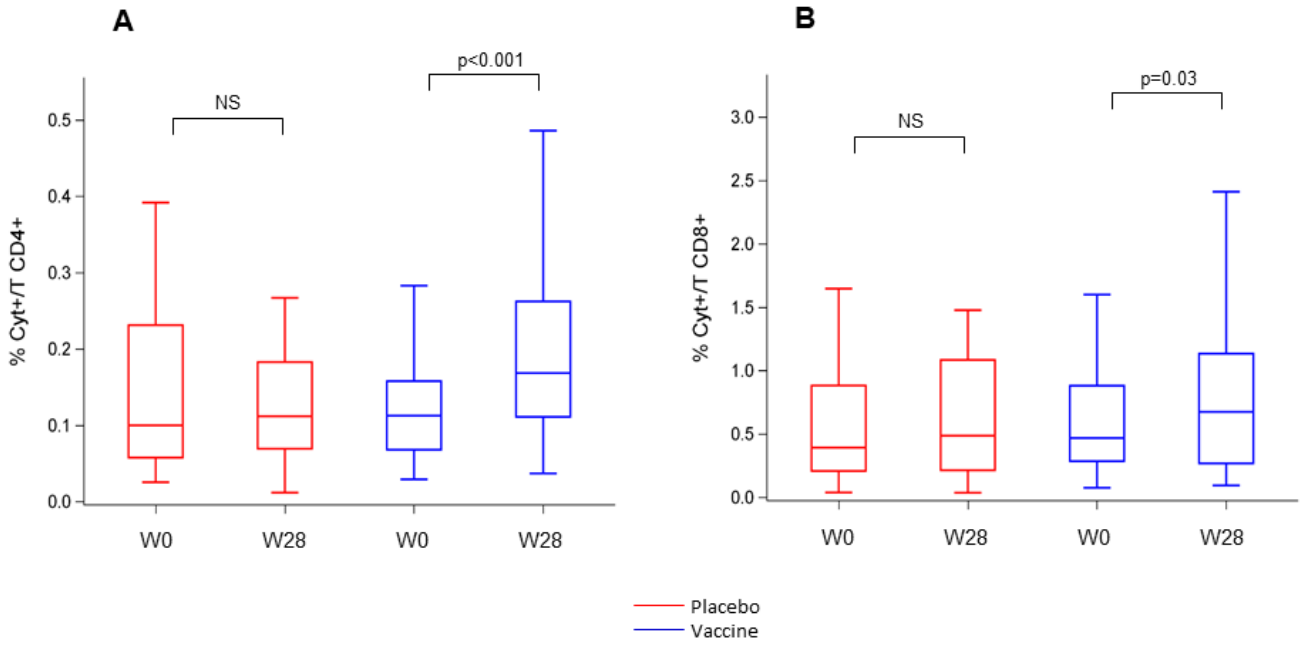

Figure 2

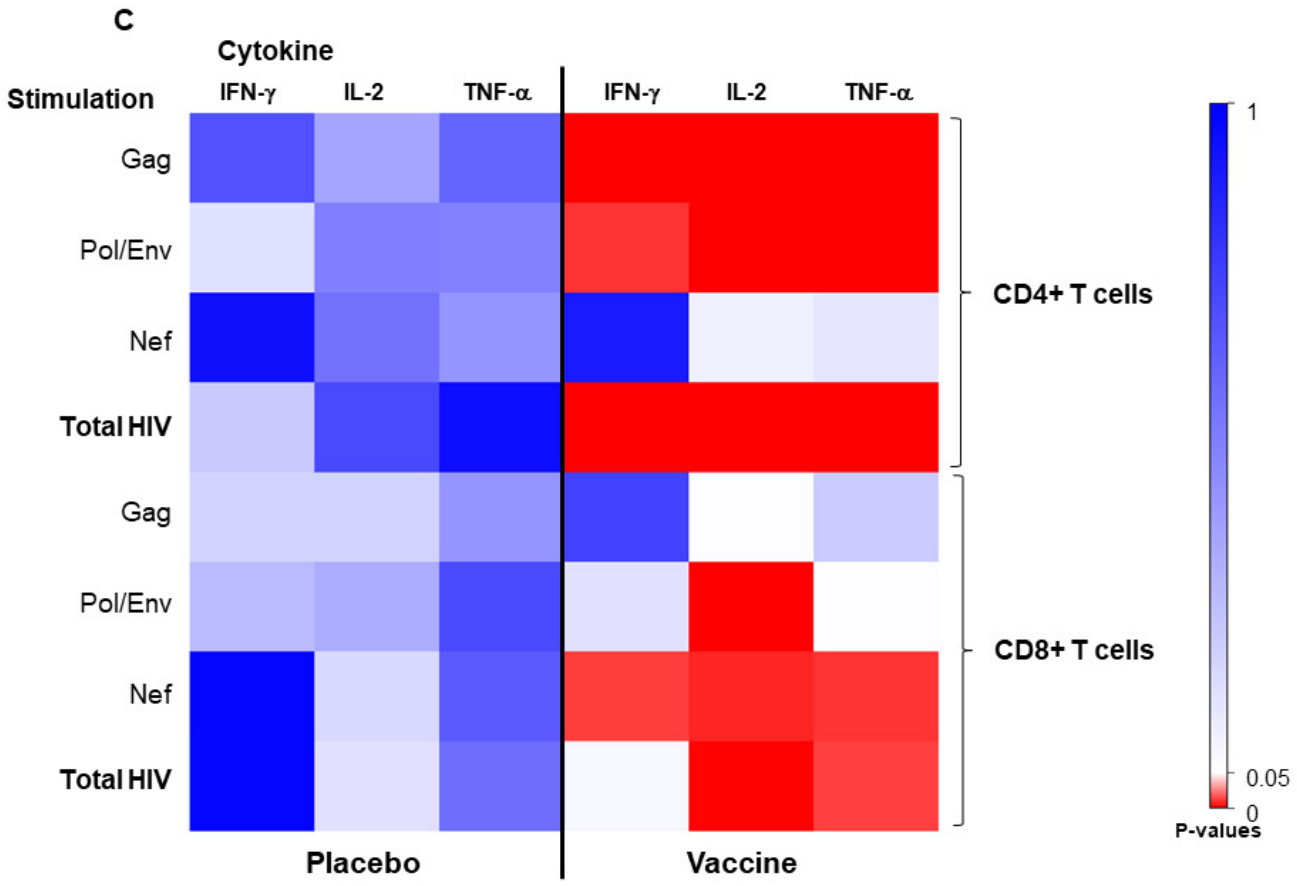

Figure 2

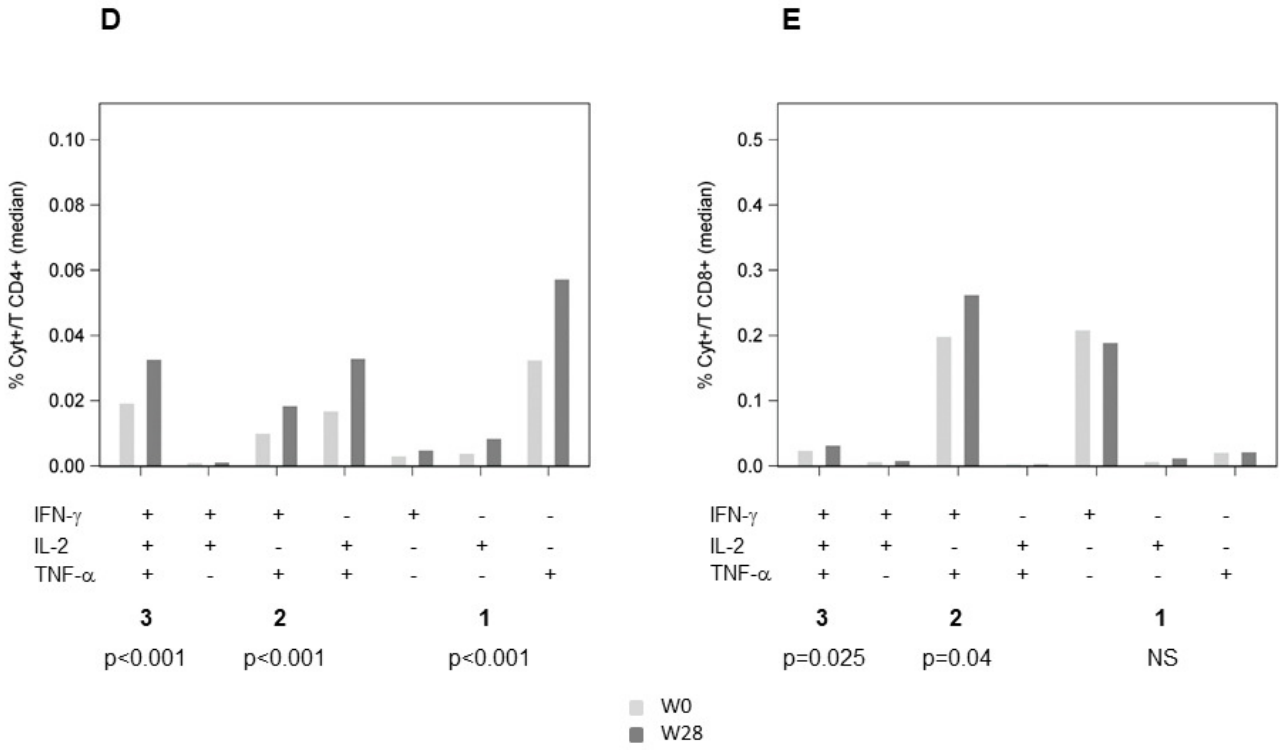

Figure 2

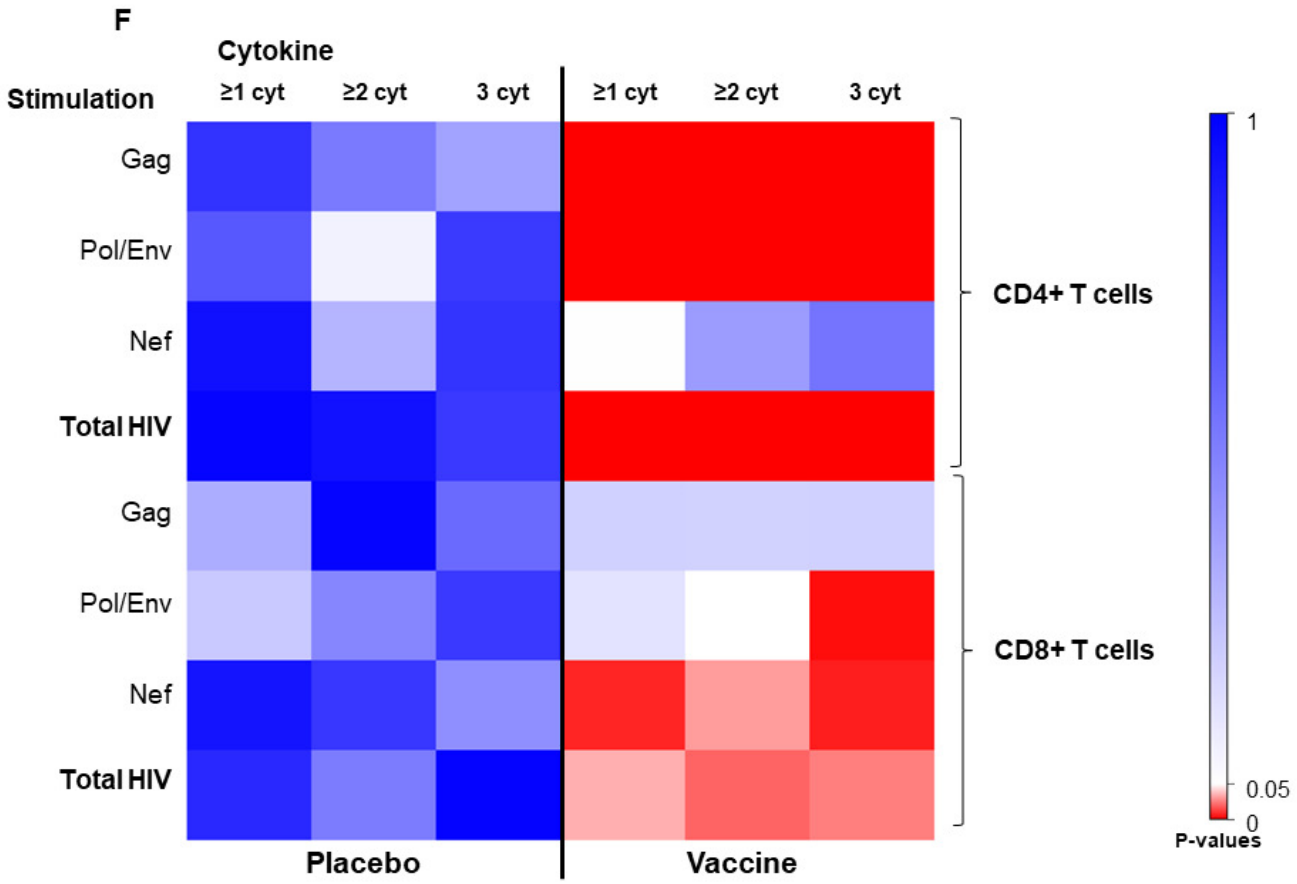

Figure 3

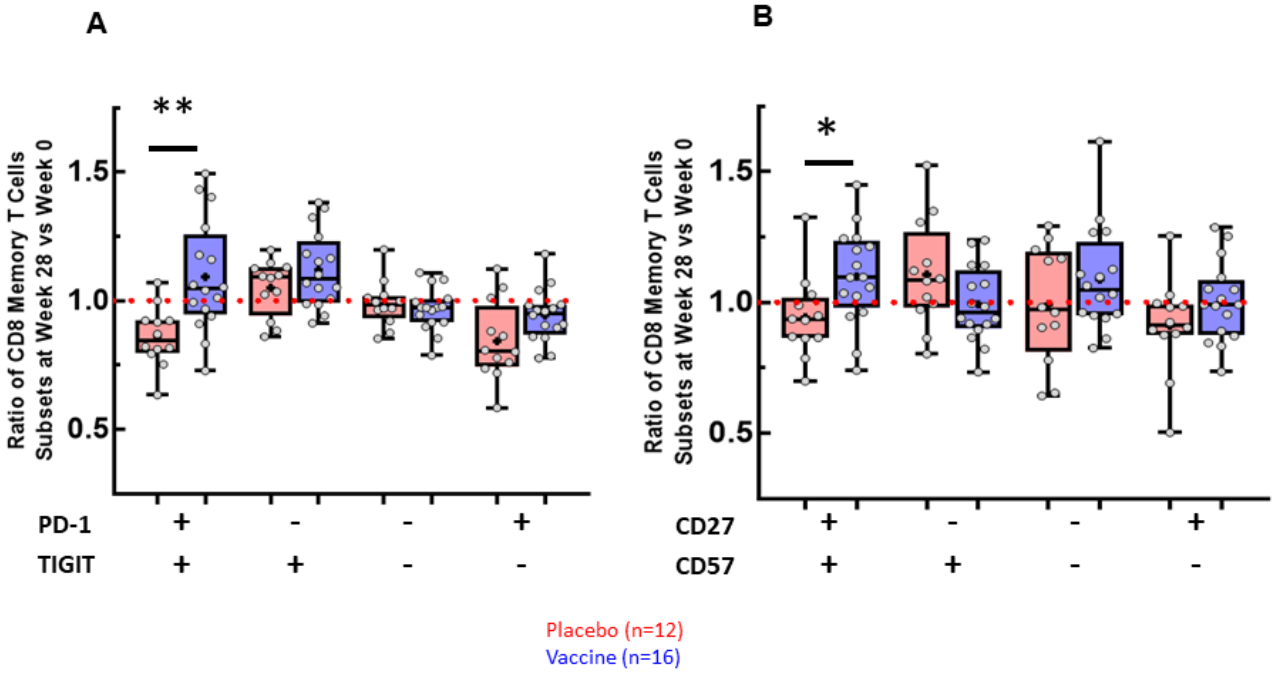

Figure 3

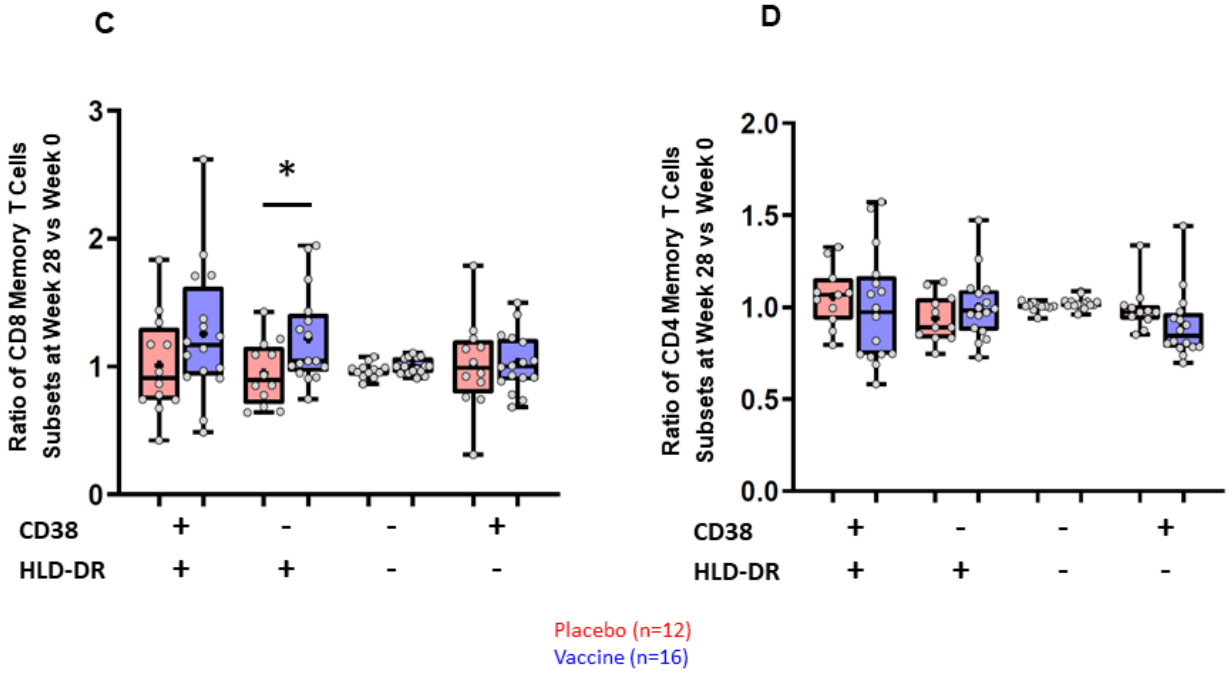

Figure 4

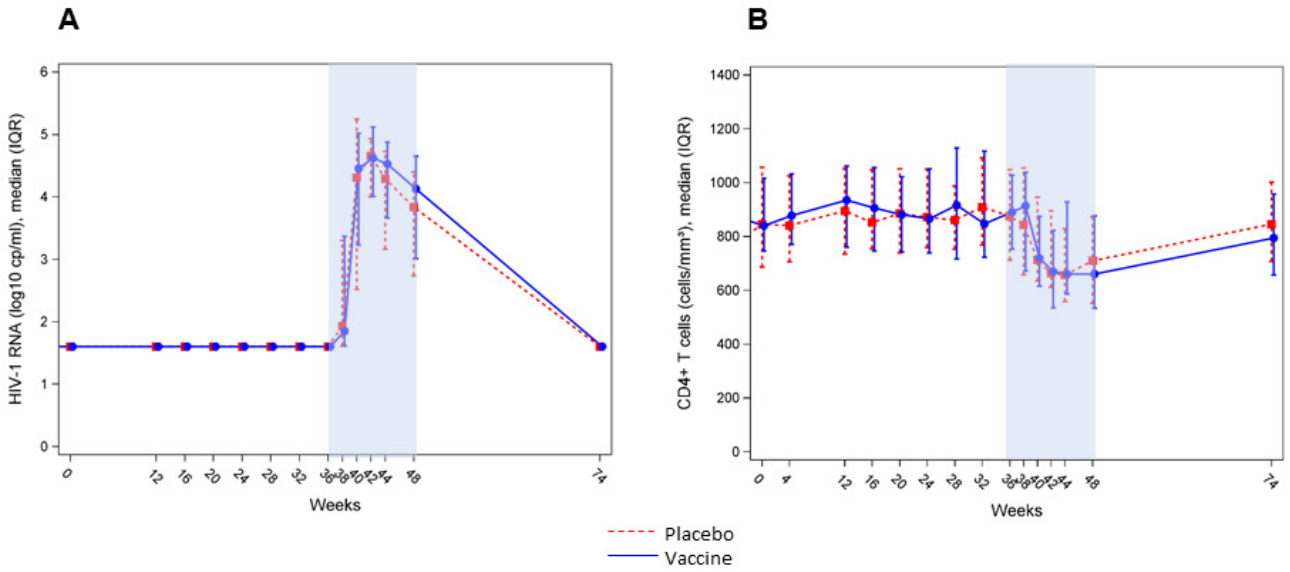

Figure 5