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

3
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36

37 **ABSTRACT**

38 In this placebo-controlled phase II randomized clinical trial, 103 HIV-1 infected
39 patients under c-ART (combined antiretroviral treatment) were randomized 2:1 to
40 receive 3 doses of DNA GTU-MultiHIV B (coding for Rev, Nef, Tat, Gag and
41 gp160) at Week (W)0, W4 and W12 followed by 2 doses of LIPO-5 vaccine
42 containing long peptides from Gag, Pol and Nef at W20 and W24 or placebos.
43 Analytical treatment interruption (ATI) was performed between W36 to W48.
44 At W28, vaccinees experienced an increase in functional CD4⁺ T cell responses
45 measured (P<0.001 for each cytokine compared to W0) predominantly against
46 Gag and Pol/Env and an increase in HIV-specific CD8⁺ T cells producing IL-2
47 and TNF- α (P=0.001 and 0.013, respectively), predominantly against Pol/Env
48 and Nef. However, analysis of T cell subsets by mass cytometry in a
49 subpopulation showed an increase of W28/W0 ratio for memory CD8⁺ T cells co-
50 expressing exhaustion and senescence markers such as PD-1/TIGIT (P=0.004)
51 and CD27/CD57 (P=0.044) in vaccinees compared to placebo. During ATI, all
52 patients experienced viral rebound with a maximum observed HIV RNA level at
53 W42 (median: 4.63 log₁₀ cp/ml; IQR 4.00-5.09) without any difference between
54 arms. No patient resumed c-ART for CD4 cell count drop. Globally, the vaccine
55 strategy was safe. However, a secondary HIV transmission during ATI was
56 observed.

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

61 **IMPORTANCE**

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

76

77

78 **INTRODUCTION**

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

91

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

98

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

102 sequences (HIV LIPO-5 vaccine) in a prime-boost regimen. These two vaccines
103 share homologous HIV sequences and strong CTL epitopes. The GTU®-
104 MultiHIV B encodes for a MultiHIV antigen (Rev, Nef, Tat, Gag p17/p24 proteins
105 and an epitope stretch of previously identified CTL epitope-rich regions encoded
106 by pol and env of a subtype B HIV-1 isolate Han-2) and lipopeptides are
107 composed of 5 synthetic peptides (Nef 66-97, Nef 116-145, Gag 17-35, Gag 253-
108 284, Pol 325-355 also from a clade B strain) to which lipid chains are covalently
109 bound. GTU®-MultiHIV B has been evaluated in HIV untreated patients where it
110 led to HIV-specific sustained CD4⁺ and CD8⁺ T cell response as well as a
111 significant decline of plasma HIV viral load (5). More recent results combining
112 transcutaneous (TC) and intramuscular (IM) injection of GTU®-MultiHIV B
113 showed a lack of improvement of immune responses in HIV treated patients and
114 concluded for the need of a combinatory approach (6). LIPO-5 has been
115 evaluated in healthy adults where it led to a sustained HIV-specific CD4⁺ and
116 CD8⁺ T-cell responses (7, 8).

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

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

127 **RESULTS**

128 **Study participants**

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

138

139 **Global overview of vaccine immunogenicity**

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

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

163 To extend the analysis of immune cells, an ancillary analysis of T cell phenotypic
164 profile was performed in 28 patients (12 placebo and 16 vaccinees) with mass
165 cytometry allowing the detection of 40 cell surface markers. Figure 3 depicts the
166 W28/W0 ratio of gated positive populations for each marker in vaccinees
167 compared to placebo. Significant changes of CD8⁺ memory T cell subsets were
168 observed in the vaccine group after vaccination with a higher frequency of
169 memory CD8⁺ T cells coexpressing PD-1 and TIGIT (Figure 3A), and
170 coexpressing CD27 and CD57 (Figure 3B). Changes in the population of CD8⁺ T
171 cell exhibiting markers of activation were also observed as memory HLA DR⁺
172 CD38⁻ CD8⁺ T cells were increased in the vaccine group (Figure 3C) without any
173 modification of the memory CD4⁺ T cells (Figure 3D). No change was observed
174 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/mm³ (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

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

237

238 **Safety**

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

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

249

250

251 **DISCUSSION**

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

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

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

271

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

275

276 The rationale to combine a DNA GTU prime and HIV long lipopeptides was
277 based on previous results obtained with each individual vaccine component.
278 Administration of DNA GTU in cART naïve individuals resulted in a modest, but
279 significant, decrease of plasma HIV viral load (up to 0.5 log₁₀ copies/ml) in a
280 large therapeutic study performed in South African individuals (5). Previous
281 therapeutic vaccine studies centered around HIV lipopeptides provided also
282 encouraging results (7, 8). Combination of ALVAC/HIV lipopeptide and IL-2
283 preceding ATI in chronically HIV-1 infected patients resulted in a greater chance
284 to maintain a viral load during a 24 weeks ATI period (HIV RNA below 10 000
285 copies/ml as predefined in the present study) compared to individuals from a
286 control arm (12, 15). In a recent non randomized vaccine study, we showed that
287 vaccination with *ex vivo* generated Dendritic Cells (DC) loaded with HIV-
288 lipopeptides (the Dalia trial) elicited strong CD4⁺ and CD8⁺ T cell responses
289 associated with a control of viral replication following ATI in chronically HIV-1
290 infected individuals (16). In these two previous studies, we found a correlation
291 between vaccine elicited responses and the magnitude of viral replication or the
292 frequency of individuals maintaining plasma HIV viral load below a predefined
293 threshold following ATI (12, 15–18).

294

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

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

388

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

391 ATI, such as a confirmed >30% decline in CD4⁺ T cell count, an absolute CD4⁺ T
392 cell count <350 cells/mm³, or the development of acute retroviral syndrome.
393 Globally the strategy was well tolerated and no individuals reached these safety
394 criteria for resuming cART.

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

405

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

414

415 **MATERIALS AND METHODS**

416

417 **Study design and participants**

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

430

431 **Randomization and masking**

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

438

439 **Procedures**

440 DNA GTU MultiHIV and HIV LIPO-5 have been described elsewhere (5–7).
441 Briefly, GTU®-MultiHIV B clade, developed by FIT Biotech, encodes for a
442 MultiHIV antigen (synthetic fusion protein built up by full-length polypeptides of
443 Rev, Nef, Tat, p17 and p24 with more than 20 Th and CTL epitopes of protease,
444 reverse transcriptase (RT) and envgp160 regions of the HAN2 HIV-1 B clade.
445 HIV-LIPO-5 vaccine consisted in 5 long HIV peptides from (Nef 66-97; Nef 116-
446 145; Gag 17-35; Gag 253-284 and Pol 325-355) to which lipid tail are covalently
447 bound. These lipopeptides which cover HIV epitopes binding to > 90% of HLA
448 molecules, permit presentation of CD4⁺ and CD8⁺ T cell epitopes as well as
449 generation of humoral immunity (17).

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

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

465

466 **Intracellular cytokine staining (ICS) assay**

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

476

477 **Mass cytometry staining and analyses**

478 In a subpopulation of patients from both groups selected among those presenting
479 an ICS response, a mass Cytometry (CyTOF) analysis was performed at weeks
480 0 and 28. PBMC were thawed, rested and then stained using metal-conjugated
481 antibodies according to the CyTOF manufacturer's instructions (Fluidigm, San
482 Francisco, CA). Cell viability staining was performed using the Cell-IDTM-103 Rh
483 Intercallator at a final concentration of 1 μ M that was incubated with PBMC for 15
484 minutes. PBMC from an individual donor were treated in parallel and multiplexed
485 for staining and mass cytometry analysis to limit sample variation due to sample
486 preparation and analysis. Multiplexed week 0 and week 28 PBMC were stained

487 for 20 minutes with either anti-CD45 89 Y or anti-CD45 194 Pt isotopes,
488 respectively, and then washed with CSM buffer (PBS, 0.5% BSA, 0.02% sodium
489 azide, all from Sigma) before combining the two samples. Pooled samples
490 containing $2-4 \times 10^6$ cells were stained for 30 minutes using a cocktail of
491 antibodies for cell surface markers in a total volume of 50 μ l (Table 5). Cells were
492 subsequently washed with CSM and PBS, fixed with 2.4% formaldehyde
493 (Thermo Fisher) in PBS for 5 minutes and then resuspended in DNA-intercalation
494 solution (PBS, 1 μ M Ir-Intercalator, 1% formaldehyde, 0.3% saponin) before
495 storage at 4°C until analysis. For CyTOF analysis, cells were washed 3 times
496 with MilliQ water and resuspended at 0.5×10^6 cells/ml in 0.1% EQ™ Four
497 Element Calibration Beads solution (Fluidigm). Samples were normalized for the
498 EQ™ bead intensities using the matlab normalizer software to limit inter analysis
499 staining intensities. Data were processed and analyzed with cytobank. Since W0
500 and W28 samples for a given donor were multiplexed and stained in parallel, the
501 relative changes in marker intensities were determined using the W28/W0 ratio
502 for the indicated gated positive populations.

503

504 **Study endpoints**

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

511 Clinical and virological secondary endpoints were as follows: the frequency of
512 clinical and biological adverse events occurring during the trial; CD4⁺ T cell
513 counts at W40, W44, W48, W74; HIV-1 RNA loads at W40, W44, W48, W74; the
514 virological success, assessed as the percentage of participants with plasma
515 HIV-1 RNA load below 10 000 copies/mL at W48 considering virological failure
516 for participants not having interrupted cART at W36; proportion of participants
517 who reinitiated ARTs after W36; proportion of participants with CD4⁺ T cell
518 counts < 350/mm³.

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

526

527 **Statistical analysis**

528 The sample size calculation was based on the assumption that a reduction by at
529 least 0.7 log₁₀ copies/mL in plasma HIV-1 RNA level at the end of the ATI in the
530 vaccinated group compared to the placebo group (standard deviation of the viral
531 load at the end of the interruption estimated at 1.0 log₁₀ copies/mL in the
532 Window/ANRS 106 trial). With a two-sided type I error of 5% and a power of at
533 least 90% (Wilcoxon-rank test), the targeted number of participants was 35 in the
534 placebo group and 70 in the vaccine group.

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

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

550 Statistical analyses were performed using SAS (version 9.3 or higher, SAS
551 Institute, Cary, NC, USA) and R (version 3.6.0, The R Foundation for Statistical
552 Computing, Vienna, Austria). Tests with a two-sided *P* value of <.05 were
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594

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596

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- 735

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)	0.878
Median (IQR)	5.26 (4.58 ; 7.00)	5.15 (4.73 ; 7.00)	5.16 (4.70 ; 7.00)	
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

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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 (%)	213 (97)		397 (98)		610 (98)	
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	

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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)
	Rheumatoid 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)

792	Respiratory, thoracic and mediastinal disorders	Pulmonary disorder, n (%)	.	1 (12.5)	1 (6.7)
793	Metabolism and nutrition disorders	Diabetes, n (%)	.	1 (12.5)	1 (6.7)
794	<hr/>				

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	141 Pr	11A9	Fluidigm	0.50
CD19	142 Nd	H1B 19	Fluidigm	0.80
ICOS	143 Nd	C398.4A	Biolegend	0.80
CD69	144 Nd	FN50	Fluidigm	0.50
CD31	145 Nd	WM 59	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	1.20
CD103	151 Eu	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	1.00
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	161 Dy	L161	Biolegend	0.30
CD32a-APC	162 Dy	APC003	Fluidigm	3.5/1.0
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	1.20
CD45RA	169 Tm	HI100	Fluidigm	0.80
CD3	170 Er	UCHT1	Fluidigm	0.40
LAG3	172 Yb	BMS	Biotechne	1.60
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

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Figure 1

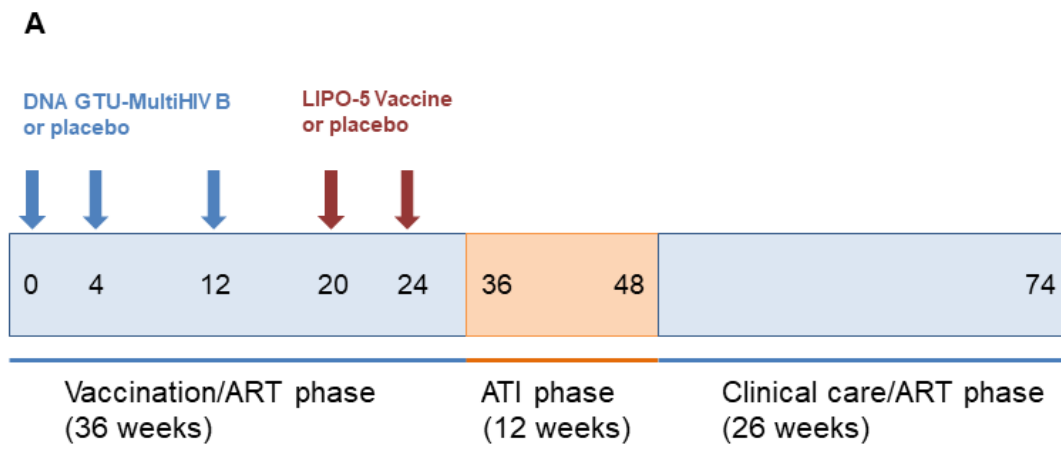


Figure 1

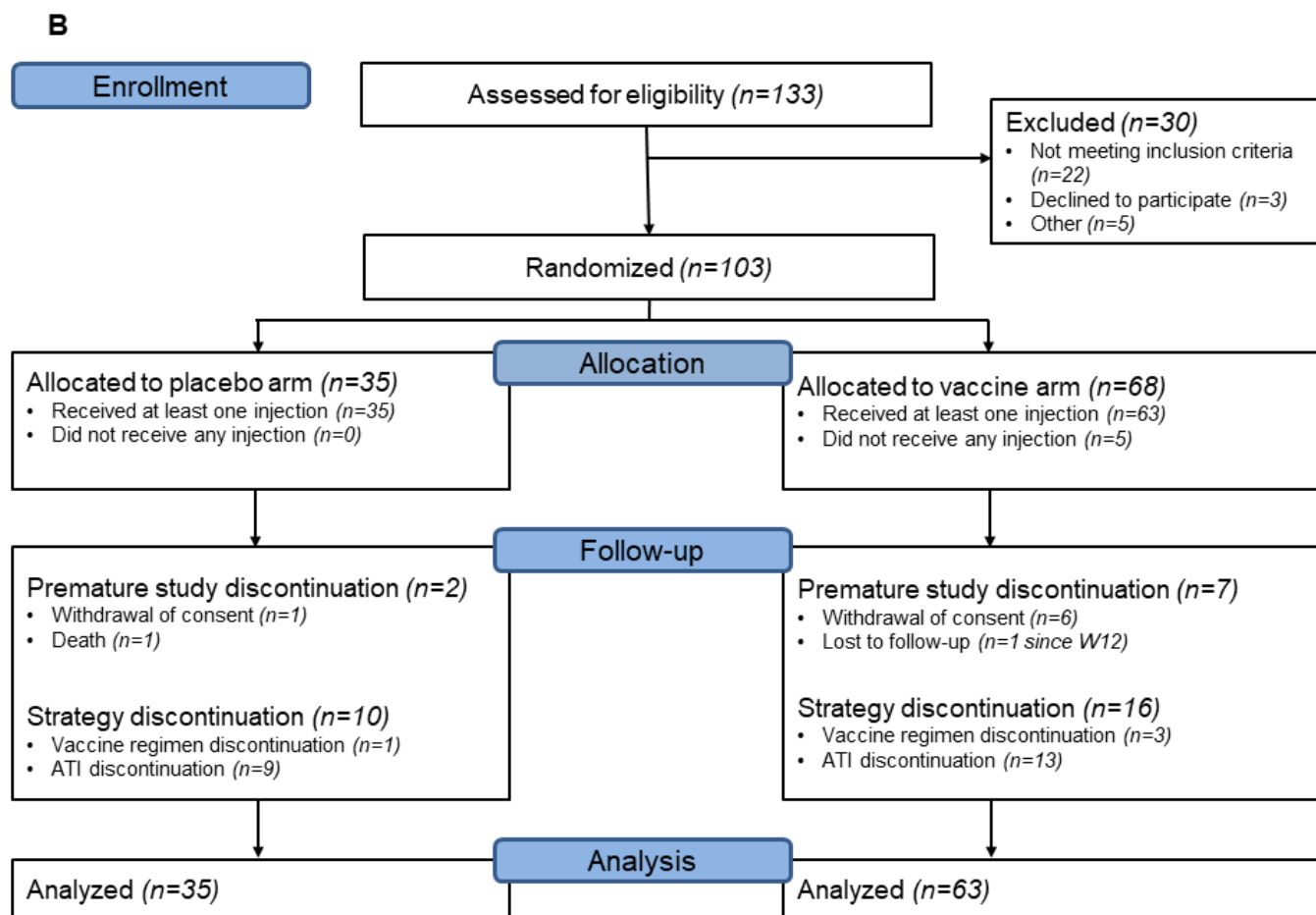


Figure 2

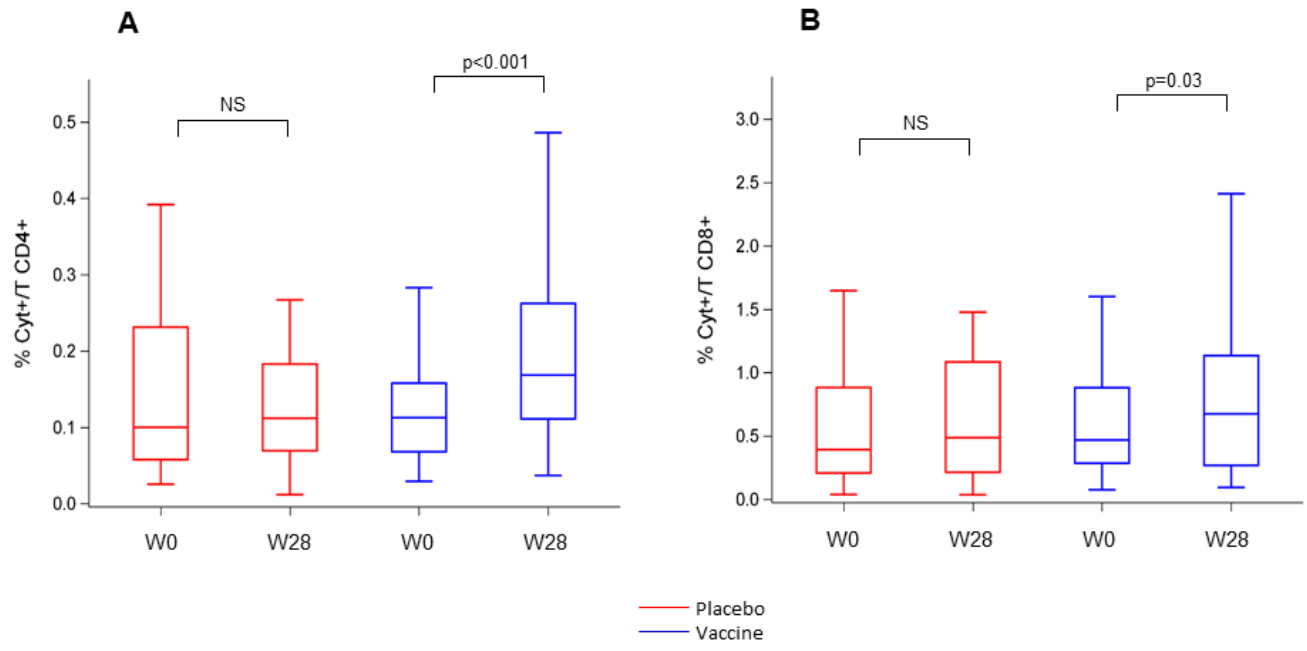


Figure 2

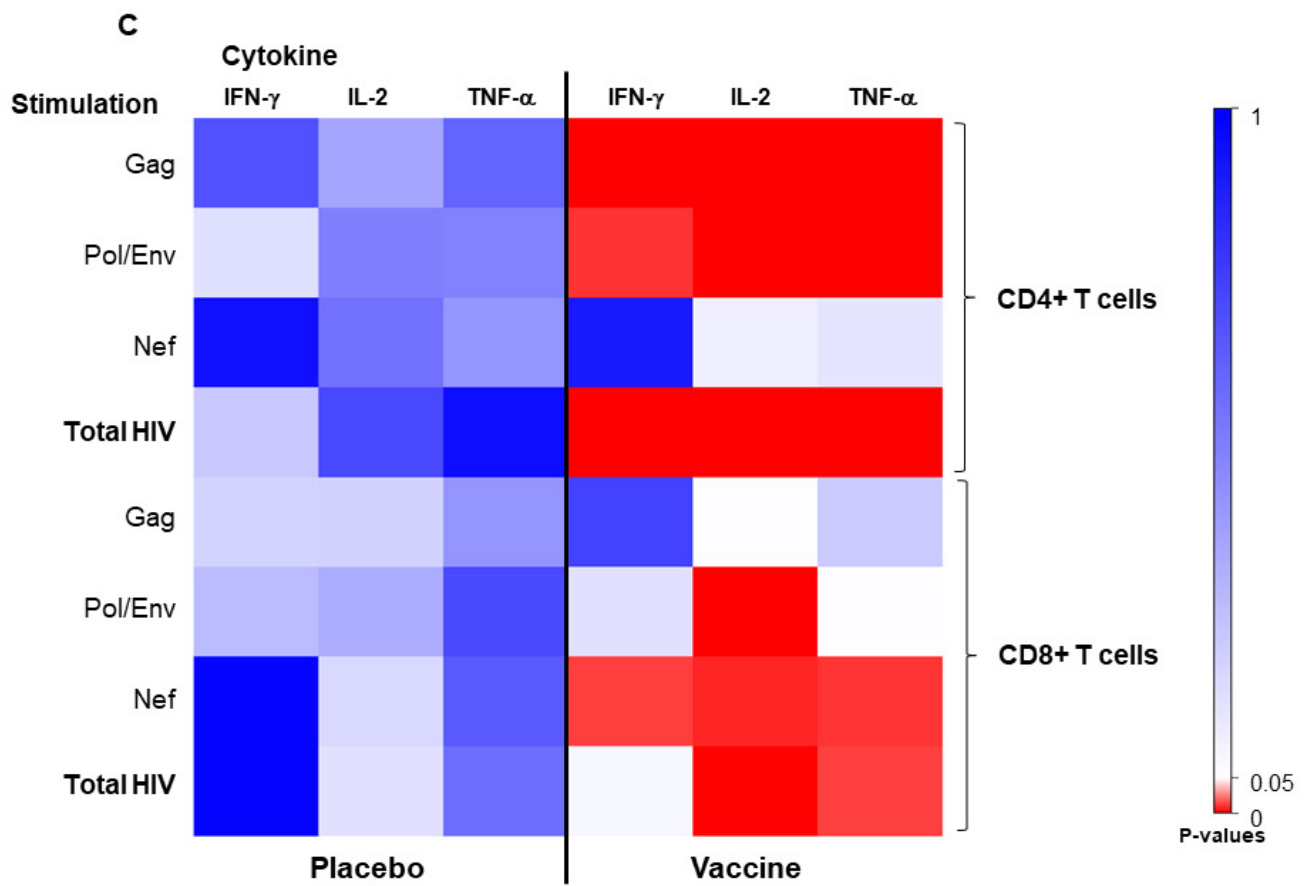


Figure 2

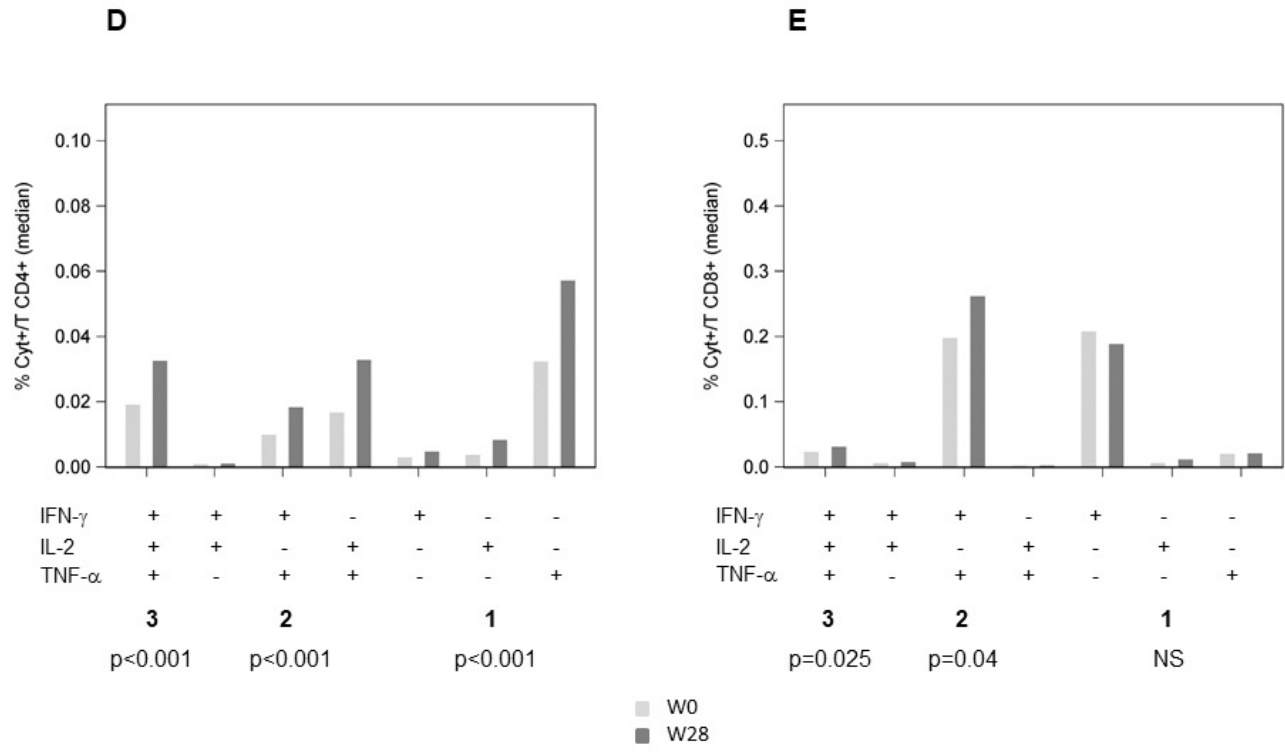


Figure 2

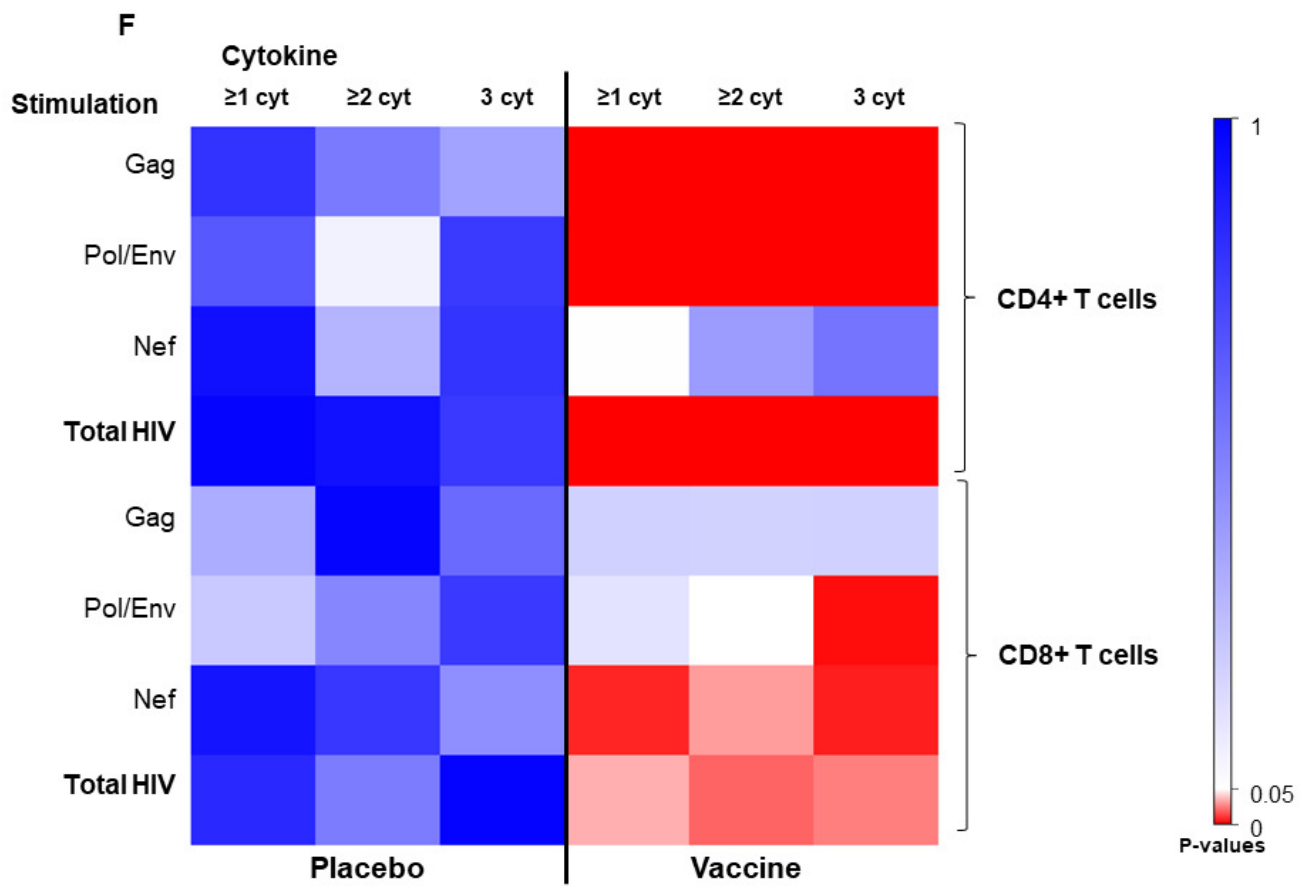


Figure 3

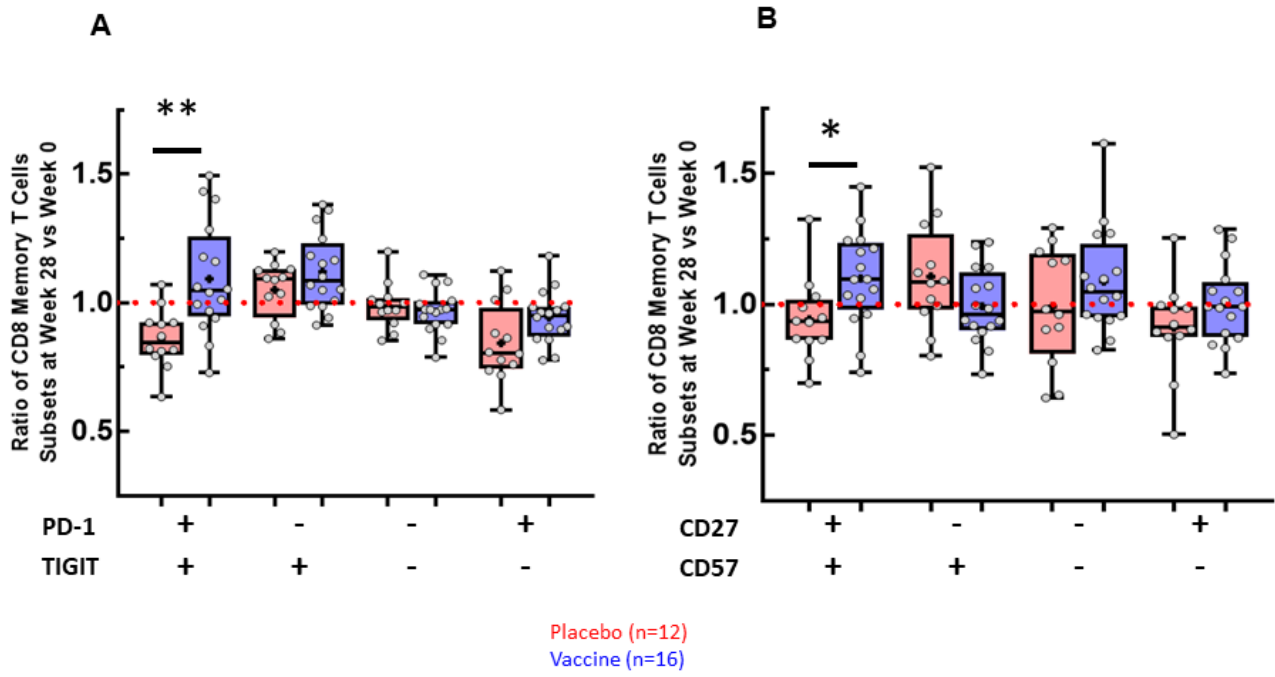


Figure 3

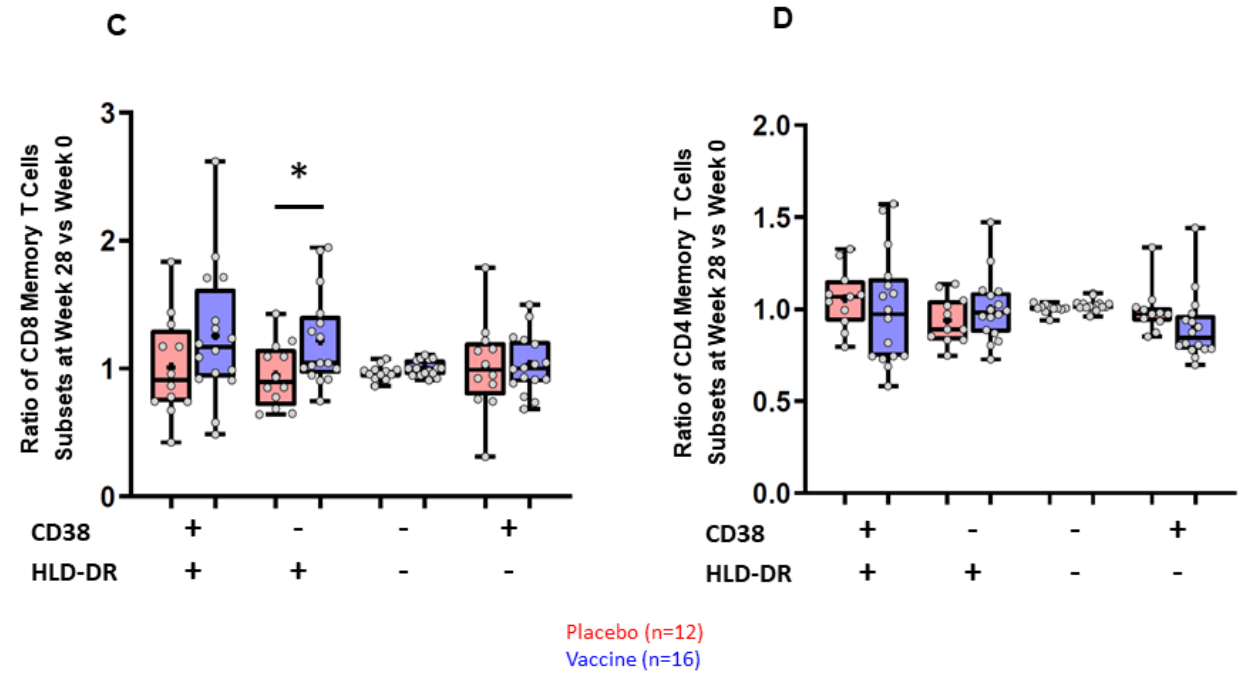


Figure 4

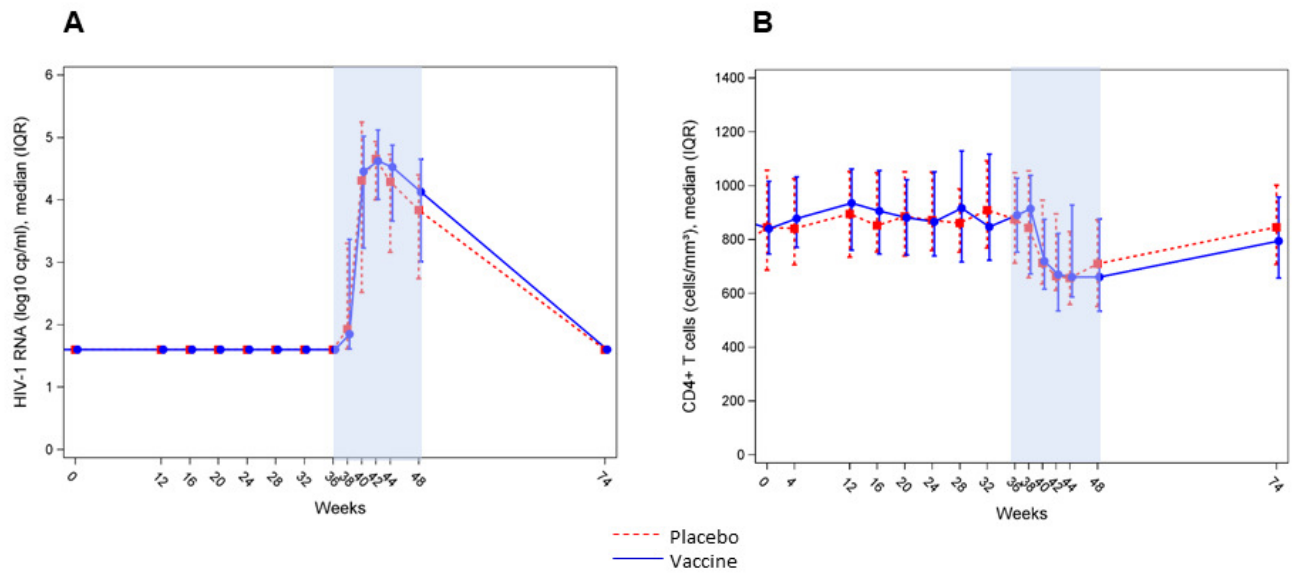


Figure 5

