Comprehensive analysis of virus-specific T-cells provides clues for the failure of therapeutic immunization with ALVAC-HIV vaccine

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Background: HIV-specific T-cell-based vaccines have been extensively studied in both prevention and therapeutic settings, with most studies failing to show benefit, and some suggesting harm. We previously performed a multicenter, double-blind, placebo-controlled phase II clinical trial in which 65 antiretroviral-treated patients were randomized to receive an HIV-1 recombinant canarypox vaccine (vCP1452) or placebo, followed by analytical treatment interruption. Patients exposed to vaccine had higher levels of viral replication and more rapid time to treatment resumption.

Objective: In the present study we report the results from extensive immunological investigations to test whether the preferential expansion of HIV-specific $CD4^+$, rather than $CD8^+$ T cells, could account for these unexpected results.

Methods: Polychromatic flow cytometry was used to characterize the functional and phenotypic profile of antigen-specific $CD8^+$ and $CD4^+$ T cells induced by the immunization.

Results: We found a significant increase in HIV-specific $CD4^+$ T cells producing IFN-γ and IL-2 in the 4 injections arm compared to the placebo arm following vaccination. In contrast, no difference was observed following vaccination in the phenotype and functional capacity within the $CD8^+$ T-cell compartment. Neither HLA biases, nor immune hyper-activation, or Env-specific facilitating antibodies were associated with the enhanced virus rebound observed in vaccinees.

Conclusion: Our data suggest that a vaccine-induced transient activation of HIV-specific $CD4^+$ but not $CD8^+$ T cells may have a detrimental effect on HIV outcomes. These findings may provide a mechanistic basis for higher rates of HIV acquisition or replication that have been associated with some T-cell vaccines.

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Introduction

Therapeutic immunization in HIV infection has been proposed as an alternative strategy to reduce viral replication, thereby limiting the length of continuous exposure to antiretroviral therapy (ART) [1]. These immune-based therapies are expected to boost or induce protective effector immune responses to levels sufficient to generate durable control of HIV-1 replication after antiretroviral treatment interruption. However, thus far success with these types of approaches has been limited. Of the candidates proposed as a therapeutic vaccine modality, the HIV-recombinant canarypox vaccine (ALVAC-HIV), expressing several HIV-1 structural and nonstructural genes, has been thoroughly investigated. Despite the fact that this immunogen did not show clinical benefit following immunization of patients at the time of primary HIV infection [2–4], a modest decrease in plasma HIV viral load and a slightly longer duration off therapy was reported in chronically infected patients who received ALVAC-HIV immunizations [5–7]. Yet, this protection was not confirmed by our group. Rather, immunization resulted in an enhancement of HIV replication after discontinuation of therapy following ALVAC-HIV immunization in chronically infected patients [8]. In fact, after treatment interruption, viral loads were significantly elevated in the two immunization arms compared to the placebo group, with increased proportions of patients requiring treatment resumption.

This enhanced viral replication following immunization has never been observed thus far and suggests that immunization may have induced some form of immune activation, which may have provided ground for viral propagation. It is well established that vaccination can transiently activate the immune system, which translates in untreated patients into transient peaks of viremia [9,10]. However, in ART-treated immunized patients, the virus usually continues to be controlled [11], and viral blips were not reported during the ALVAC-HIV immunization phase in this particular study [8] and in others [5,12]. In contrast, elevated viral replication, as observed in our study, argues for a role of immunization in viral enhancement, raising the question of whether the immune responses elicited by this vaccine may have been more detrimental than protective. However, recently a trial using the vCP1521 vector in combination with AIDSVAX B/E showed a modest but significant reduction in the rate of HIV infection in a large phase III preventive trial, although no immune correlates of protection have been defined thus far [13].

In an effort to further understand the unanticipated results from the MANON-02 vaccine trial, we conducted in-depth immunological investigations to define parameters that may underlie enhanced viral replication in vaccinees. We tested whether the enhanced virus replication observed in the immunization arms reflected three non-exclusive hypotheses: a bias in patient sampling may have limited vaccine efficacy or virus control and might reflect some imbalance in the patients HLA alleles; the vaccine was not sufficiently immunogenic and induced $CD4^+$ T-cell responses in the absence of protective $CD8^+$ T-cell responses, thereby augmenting target cell availability unabated by cytotoxic T-cell control; or the vaccine itself or the trial design (i.e. treatment interruption) induced T-cell activation might have increased target availability.

Methods

Study participants

As described [8], 65 patients were randomized in a multicenter, double-blind, placebo-controlled phase II clinical trial to receive 3 or 4-vaccine injections or placebo. Patients were proposed to discontinue therapy 1 month after the last immunization (W24) if their virus was still undetectable (supplementary table, http://links.lww.com/QAD/A93). The protocol was approved by the independent Ethics Committees at which the study was conducted. All patients provided written informed consent.

Vaccine

vCP1452 is a recombinant canarypox virus vaccine (Sanofi Pasteur, Marcy l'Etoile, France) expressing the HIV-1 MN gp120, gp41, p55 gag polyprotein, the protease, and RT and Nef CTL epitopes from the LAI strain [3,12,14]. Vaccine ($10^{7.08}$ per dose) or placebo (saline solution) was injected intramuscularly.

IFN-γ ELISpot assay

ELISpots were performed on cryopreserved peripheral blood mononuclear cells (PBMCs) (viability $\geq$85%) from 56 study participants. Forty-seven patients interrupted ART 1 month after immunization. No difference was observed with respect to CD4 nadir and CD4 cell count among the 47 patients studied (median CD4 nadir = 240 cells/μl and CD4 count = 624 cells/μl). Pools of 10–11 15-mer synthetic HIV-peptides were generated spanning HIV-1 LAI-Gag, RT, and Nef vaccine sequences (Sigma, St Louis, Missouri, USA) [8,15]. Unstimulated cells served as a negative control, and positive controls included PHA (Abbott Laboratories, Abbott Park, Illinois, USA). The positivity threshold was 50 spot forming cell (SFC)/million PBMCs after background subtraction.

Flow cytometry and reagents

The functional profile and phenotype of HIV-specific $CD8^+$ T cells was defined in 47 study patients: 17, 14, and 16 ALVAC 4 or 3-injections and placebo, respectively. These patients were selected based on strong IFN-γ ELISpot responses (above 100 SFC/10^6 PBMCs). Analyses were performed at baseline (W0); 4-weeks post

second or third injection (ALVAC 3 or 4, respectively) (W12), and 1-month after the last immunization (W24). The patients were not significantly different from the whole study group (median CD4 nadir = 246 cells/μl, CD4 count = 619 cells/μl). Only 21 patients interrupted ART (8 ALVAC 4-injections, 5 ALVAC 3-injections, and 8 placebo controls). Analyses were performed 1 and 3 months after treatment interruption (W28 and W36).

The following antibodies were used for the analyses: CD3 (Pacific-Blue or PercP-Cy5.5), CD4 (Alexa-Fluor 700), CD8 (allophycocyanin [APC]-Cy7 or Alexa-Fluor 700), CCR7 (phycoerythrin [PE]-Cy7), CCR5 (APC-Cy7), CD27 (APC), CD28 (PE-Cy5), CD38 (APC or PE), CD69 (PE-Cy5), CD57 (FITC), PD-1 (fluorescein-isothiocyanate [FITC]), Ki67 (PE), CD107a (PE-Cy5), CD40L (PE or FITC), IL-2 (APC or PE), IFN-γ (Alexa-Fluor 700 or FITC) and TNF-α (PE-Cy7), BD-Biosciences, San Jose, California, USA; CD4 (ECD), CD45RA (ECD), Beckman-Coulter, Fullerton, California, USA; CCR9 (PE), MIP-1β (FITC), R&D Systems; CD103 (Alexa-Fluor 647), eBioscience; and CD27 (Alexa-Fluor 700), HLA-DR (APC-Cy7), CXCR4 (PE-Cy5), CLA (FITC), BioLegend. HLA-A*0301 RY10, pentamer was purchased from ProImmune; HLA-A*0201 SL9, HLA-A*0201 IV9, HLA-B*0702 GL9, HLA-B*0702 RL9, HLA-B*0801 FL9, HLA-B*0801 EI8 tetramers were synthesized as described [16]. PBMCs were stained as described [17]. Cells were analyzed on an LSRII (BD-Biosciences) to perform eight-color to nine-color flow cytometry, and the data were analyzed using FlowJo software (version 8.2; TreeStar, Inc., Ashland, Oregon, USA). Multifunctional data were analyzed with the softwares PESTLE (version 1.5.4) and SPICE (version 4.1.5; obtained from M. Roederer, NIH, Bethesda, Maryland, USA). Percentage frequencies of multifunctional cells were calculated within the total population of detectable antigen-specific $CD8^+$ T cells.

Cell stimulation for multifunctional assay

Thawed and rested PBMCs (≥85% viable) were incubated with peptide pools, or specific peptides (prestained with titrated pentamer/tetramer), anti-CD28/CD49d (BD-Biosciences), and anti-CD107a antibody for 6 h in the presence of Monensin and Brefeldin-A (Sigma-Aldrich, St Louis, Missouri, USA) [18]. Medium alone served as a negative control. Fix-&-Perm buffer (Invitrogen, Paisley, UK) was used according to the manufacturers' recommendations to permeabilize cells prior to staining for intracellular markers. Staining for extracellular markers was performed before the fixation/permeabilization steps to minimize detrimental effects of the fixation on cell surface receptors.

Neutralizing and enhancing-antibody activity

Both neutralizing and enhancing-antibody were analyzed employing four viral strains belonging to various clades, as described [19]. Heat-inactivated sera collected from 26 representative patients (median CD4 nadir = 262 cells/μl and CD4 count = 623 cells/μl) at W28 were tested using serial two-fold dilutions of the plasma (from 1 : 10 to 1 : 80) as described [19,20].

HLA typing

HLA-typing was carried out by LABType SSO (sequence specific oligonucleotide) Class I (Locus A and Locus B) assay (InGen BioSciences, Chilly Mazarin, France) DNA typing method.

Statistical analysis

The HIV-RNA values were $\log_{10}$ transformed before analysis. All data were compared between vaccine and placebo groups with the Mann–Whitney U test. A Benjamini and Hochberg correction was used to account for multiple comparisons [21].

Results

Lack of influence of HLA background on enhanced HIV replication after immunization with ALVAC-HIV

To determine whether the distribution of protective vs. nonprotective HLA-class I alleles, among the vaccine and placebo arms, was associated with differential viral control following treatment interruption, all patients were HLA-class I genotyped. Among the 54 patients in whom ART was interrupted, three patients encoded a protective HLA-class I allele in the placebo arm [22], including one patient with HLA-B27 and two with HLA-B57. In contrast, patients in the immunization arms did not encode any of the alleles associated with protection from disease progression. Among the patients with protective alleles, the virus rebounded in all three cases, but none of these patients met the criteria for therapy re-initiation. In contrast, HLA-B35, associated with accelerated disease progression [22], was observed in seven patients, all randomized to one of the vaccine arms: four patients in the 3-injection arm and three in the 4-injection arm. The virus rebounded at high levels in all seven patients, and five patients met the criteria to restart therapy. At week 36, in the 54 patients who interrupted treatment, the median plasma viral load was significantly higher in the 4-injection ($n = 19$, $P = 0.023$) and 3-injection ($n = 20$, $P = 0.009$) arms compared to placebo ($n = 15$): 4.76 (4.27–4.98), 4.82 (4.53–5.11), and 4.40 $\log_{10}$ (4.11–4.53) copies/ml, respectively. Accounting for HLA distribution did not alter the study findings, as vaccination was still associated with higher virus levels at W36 in the immunized arms compared to the placebo with a median plasma viral load of 4.76 $\log_{10}$ (4.27–4.96) ($P = 0.042$ vs. placebo), 4.82 (4.62–5.11) ($P = 0.020$ vs. placebo), and 4.50 (4.18–4.54) in the 4-injection ($n = 16$), 3-injection ($n = 16$), and placebo ($n = 12$) arms, respectively.

Table 1. Numbers of HIV-specific T-cell responses assessed by ELISpot and plasma viral load during treatment interruption by vaccine arm.

	4 ALVAC-HIV ($n=16$) Mean (SD)	3 ALVAC-HIV ($n=17$) Mean (SD)	Placebo ($n=14$) Mean (SD)	4 ALVAC-HIV vs. placebo P	3 ALVAC-HIV vs. placebo P
W24–W0	430 (621)	291 (1187)	17 (518)	0.052	0.336
W24	1546 (1217)	1392 (1472)	1326 (1570)	0.170	0.921
W36	3730 (2729)	3114 (2878)	2431 (2038)	0.257	0.710
W36–W24	2183 (2052)	1722 (1999)	1105 (1547)	0.114	0.393
W36 viral load	4.65 (0.66)	4.62 (0.77)	4.06 (0.69)	0.013	0.003

Moderate increase in the breadth of the antigen-specific T-cell repertoire after immunization with ALVAC-HIV

Previously, we showed a significant increase in the frequency of HIV-specific IFN-γ producing T cells, in all patients receiving four ALVAC-HIV vaccine [8]. At baseline, approximately 66% of the ELISpot responses were directed against Gag, with a median of 343 (122–986) ($P=0.628$ vs. placebo), 159 (100–555) ($P=0.158$ vs. placebo), and 303 (198–423) in the four vCP1452 injection, three vCP1452 injection, and placebo arms, respectively. At the end of immunization, we observed a net gain of 231 (interquartile range: 2–644) Gag-specific SFC/million PBMCs ($P=0.078$) compared to baseline in the 4-injections arm, but the augmentation of these responses was not statistically significant. The three vaccine injections induced only minimal changes in HIV-specific T-cell responses at W24 compared to placebo [92 (−58 to 375) vs 5 (−76 to 152) Gag-specific SFC/million PBMC, respectively; $P=0.290$]. We next examined whether ALVAC-HIV immunization could induce the expansion of the breadth of antigens recognized by HIV-specific T-cells. Compared to placebo, a slight, but significant, increase in the number of HIV peptide pools recognized was observed after 4 (+1.5 peptide pools, $P=0.006$) but not after 3 vaccine injections.

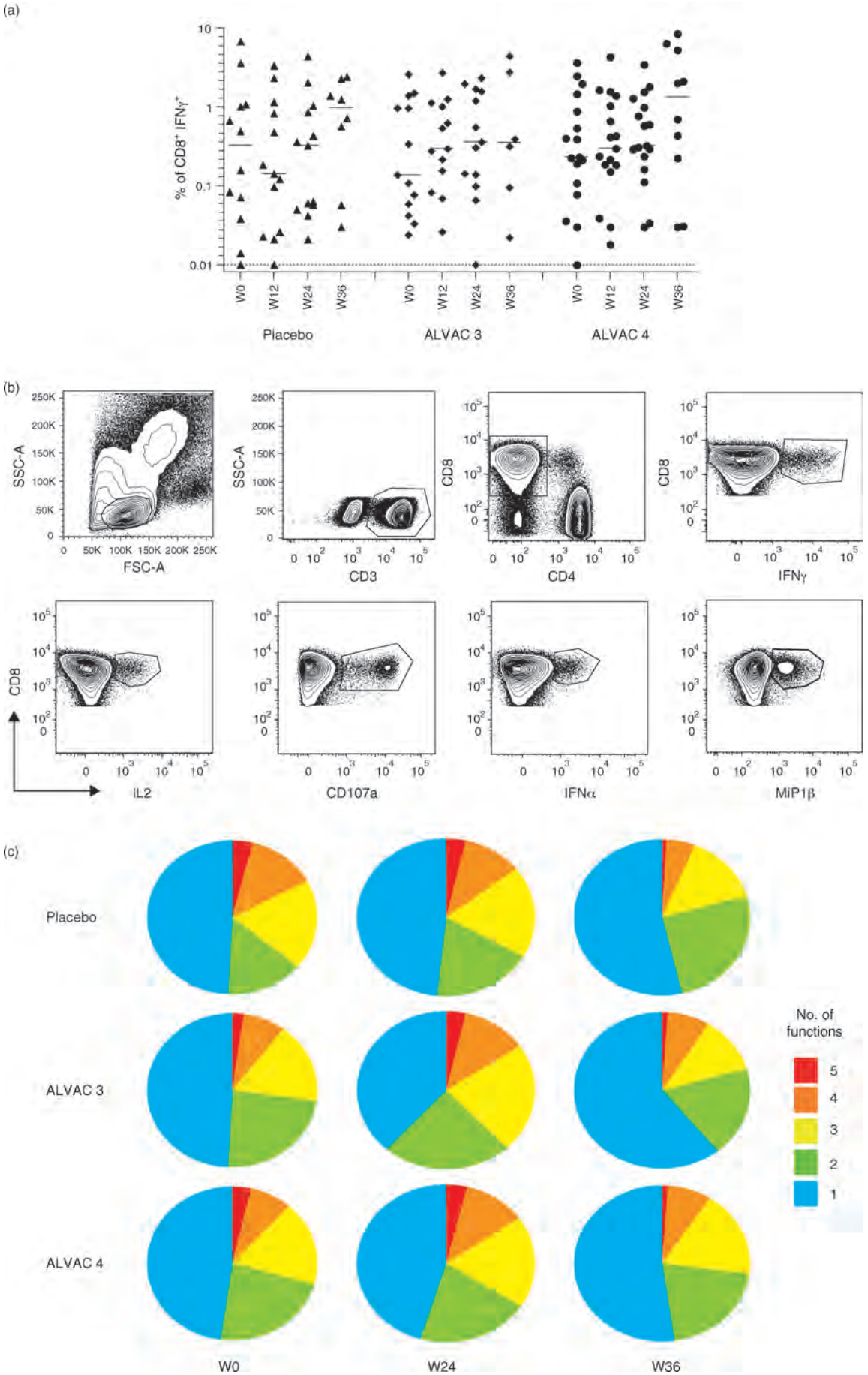
We then analyzed whether immunization differentially favored a broader HIV-specific T-cell response after viral rebound, following treatment interruption. At week 36 following treatment interruption, discontinuation of therapy resulted in a more robust boost of HIV-specific T-cell responses than immunization alone in the three arms (Table 1). Yet, the magnitude of these T-cell expansions did not differ from placebo or any significant alteration in the breadth of the response. However, plasma viral loads reached significantly higher levels in the two immunization arms compared to the placebo arm at W36, however no correlation was observed between immune responses and viral loads.

Lack of significant expansion of HIV-specific $CD8^+$ T-cell responses after ALVAC-HIV immunization

We next performed a longitudinal characterization of the functional profile and phenotype of HIV-specific $CD8^+$ T-cell responses among the vaccine groups. At baseline the frequencies of $CD8^+$ T cells producing IFN-γ did not differ significantly among the groups ($P=0.936$ and $P=0.983$ ALVAC 3 and ALVAC 4-injections vs. placebo, respectively), in line with the IFN-γ ELISpot results [8]. However, upon immunization, we observed a modest increase in the frequency of IFN-γ producing $CD8^+$ T-cells, from 0.137% to 0.363%, and from 0.232% to 0.324%, in the 3 and 4-injection arms, respectively. However, the change from baseline compared to placebo was not statistically significant in either vaccine groups ($P=0.87$ and $P=0.81$) (Fig. 1a). In contrast, HIV-specific $CD8^+$ T cells expanded robustly following plasma viral load rebound after treatment interruption, attaining much higher levels than after immunization (Fig. 1a). Despite this expansion, there was no significant difference between immunization and placebo groups.

Qualitative, rather than quantitative, differences in T-cell activity (Fig. 1b) have become increasingly regarded as a critical determinant of antiviral efficacy for protection against pathogenic organisms [23–25]. We speculated that the ALVAC-HIV vaccine may have modulated the quality, rather than the quantity, of HIV-specific $CD8^+$ T-cell responses, in spite of the lack of a boost in the magnitude of the response. During the immunization phase, we observed minor changes in T-cell polyfunctional profiles in the vaccine groups; however this was not significant when compared to the placebo arm (Fig. 1c). Following treatment interruption, we observed a generalized decrease in the proportion of polyfunctional

Fig. 1. Characterization of HIV-specific $CD8^+$ T-cell responses. (a) IFN-γ^+ HIV-specific $CD8^+$ T-cell responses were assessed during the immunization phase (W0, W12, and W24) and treatment interruption (W36) in the three vaccine arms: placebo, ALVAC 3, and ALVAC 4. Horizontal bars show medians. The dotted line represents the positive limit of detection for intracellular cytokine staining. (b) Representative example of 'gating strategy' used to characterize the polyfunctional profile of HIV-specific $CD8^+$ T cells. Following stimulation with peptide pools, PBMCs were stained simultaneously for CD107a, IFN-γ, TNF-α, IL-2, and MIP-1β and analyzed using nine color flow cytometry. (c) The pie charts depict the background adjusted longitudinal polyfunctional profile (1–5 functions) of HIV-specific $CD8^+$ T-cell responses.



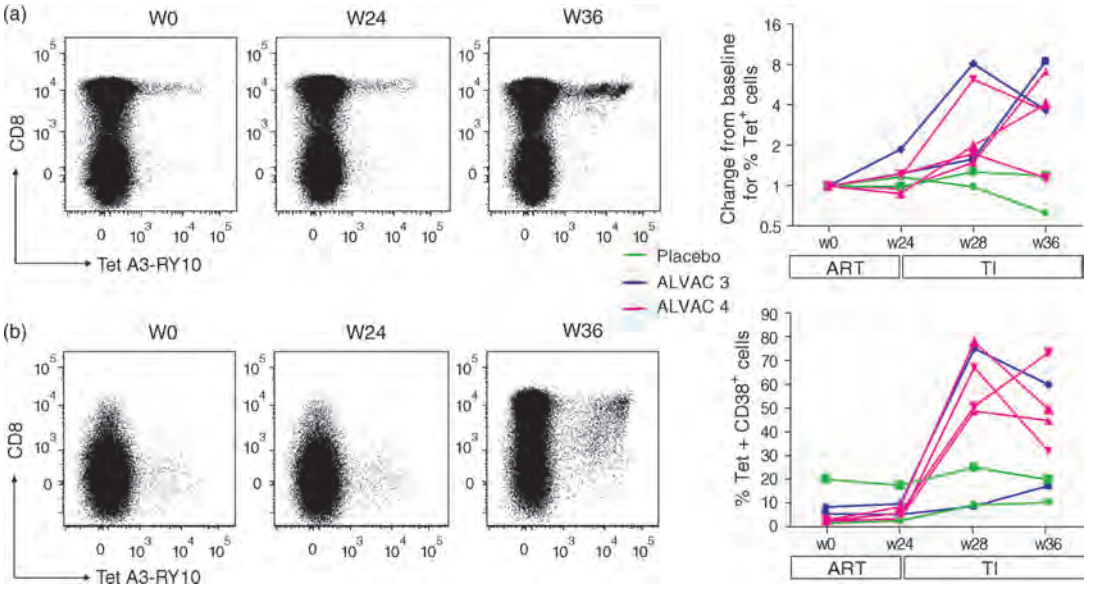


Fig. 2. Longitudinal evolution of the frequency of HIV-specific tetramer positive CD8$^+$ T cells and their activation status. (a) Representative example of an A3-RY10 tetramer staining and frequency of tetramer positive populations in different patients during the immunization phase and treatment interruption. (b) Representative example of an A3-RY10 tetramer$^+$ response with CD38 co-staining and the evolution of CD38 expression on tetramer positive populations in different patients during the immunization phase and treatment interruption.

T cells, although these changes were not significantly different between the immunization and placebo arms at all time points tested (Fig. 1c).

HIV-specific CD8$^+$ T cells secreting IFN-γ or IL-2 were largely CD27$^+$/CD28$^-$ or CCR7$^-$/CD45RA$^-$ [26–28]; yet we did not observe any difference in the memory status of responding CD8$^+$ T cells among the arms, as well as no change in the memory phenotype following vaccination compared to the baseline phenotype (data not shown).

Finally, in-depth analysis was performed on tetramer$^+$ CD8$^+$ T cells in eight patients (two, two, and four in the placebo, 3 and 4-injection arms, respectively) selected according to their HLA type. We did not notice any effect of immunization on the frequency of HIV-tetramer$^+$ CD8$^+$ T cells (Fig. 2a), activation status (i.e. CD38 expression) (Fig. 2b), or polyfunctional profile (data not shown) at W0 and W24. In contrast, tetramer-specific CD8$^+$ T cells expanded significantly after treatment interruption, were significantly activated, and expressed higher levels of CD38 (Fig. 2a and b).

ALVAC-HIV significantly augments HIV-specific CD4$^+$ T-cell responses

We next analyzed the influence of ALVAC-HIV immunization on HIV-specific CD4$^+$ T-cell responses in the same subset of donors as above. In contrast to our observations on CD8$^+$ T cells, IFN-γ producing CD4$^+$ T cells were significantly more frequent in the 4-injection arm (but not in the 3-injection arm) during the immunization phase (from 0.013 to 0.030%) compared to placebo ($P < 0.0001$) (Fig. 3a). Similar results were also obtained with IL-2 producing CD4$^+$ T cells (from 0.017 to 0.036%, $P = 0.035$) (Fig. 3b). During treatment interruption, we observed a generalized decrease in HIV-specific CD4$^+$ T-cell responses, particularly profound in the 4-injections group, with a predominant loss of IL-2-producing CD4$^+$ T cells (from 0.036 to 0.015%) (Fig. 3b).

During the immunization phase, we observed an increase in the proportion of polyfunctional CD4$^+$ T cells, particularly in the 4-vaccine group, yet this difference was not statistically significant when compared to the placebo arm (Fig. 3c). Of note, after treatment interruption, these polyfunctional CD4$^+$ T cells were lost rapidly, and appeared to be preferentially depleted (Fig. 3c).

ALVAC-HIV induced T-cell activation and target cell susceptibility

We next speculated that immunization may have modulated the activation profile and HIV-co-receptor expression on the surface of CD4$^+$ T cells rendering them differentially susceptible to infection, resulting in a more profound depletion of CD4$^+$ T cells in vaccinees. Thus, to define the effects of immunization and treatment interruption on the activation profile and HIV-co-receptor expression, we analyzed a series of markers associated with T-cell activation or viral entry on total CD4$^+$ T cells. The expression levels of CD103 and CCR9, homing markers for the gut-associated lymphoid tissue (GALT), were not or only marginally altered during the course of the intervention (Fig. 4). Although no

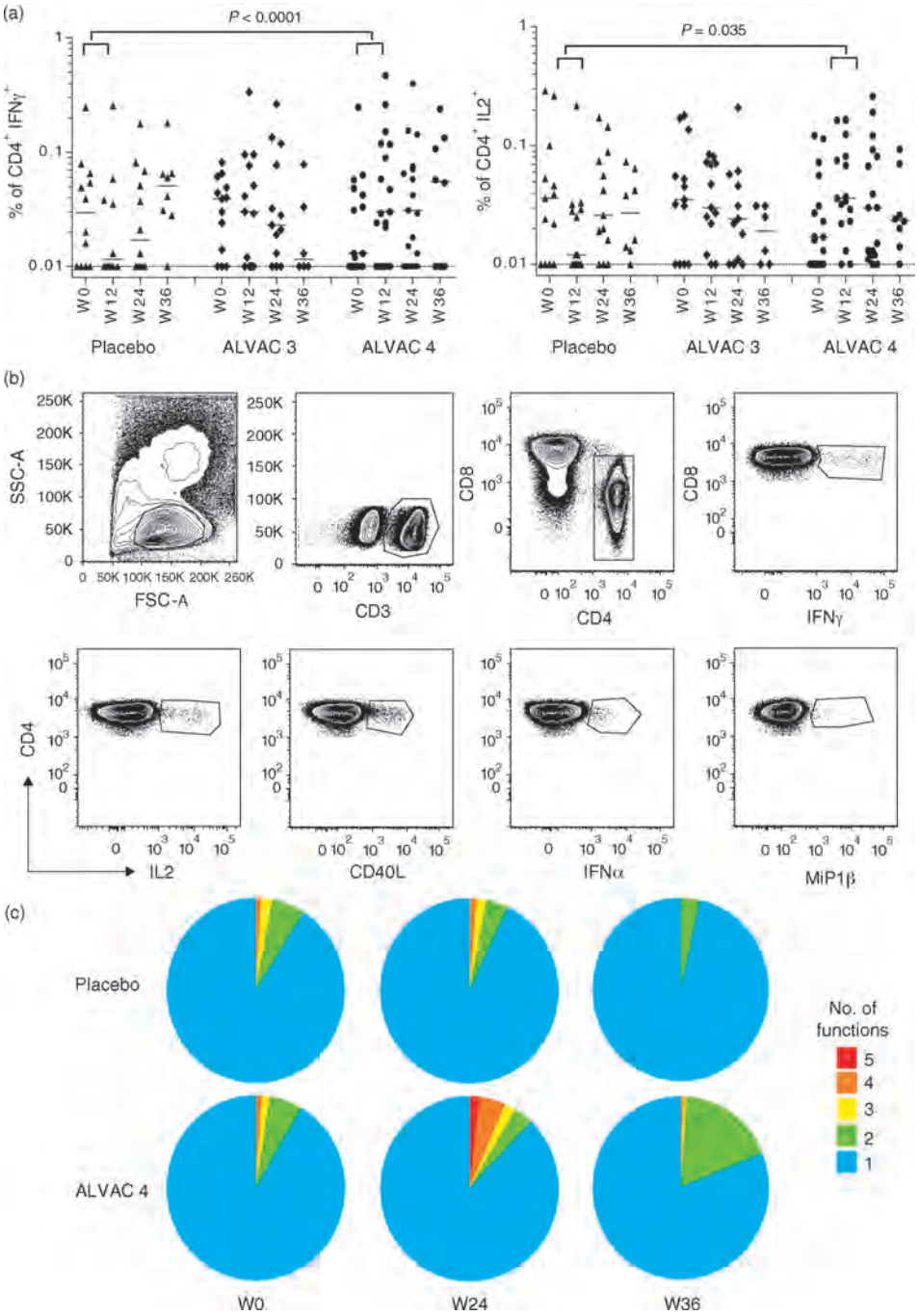


Fig. 3. Longitudinal analysis of HIV-specific CD4$^+$ T cells. (a) IFN-γ or IL-2 producing HIV-specific CD4$^+$ T-cell responses were assessed during the immunization phase (W0, W12, and W24) and treatment interruption (W36) in the three vaccine arms: placebo (closed triangle), ALVAC 3 (closed diamond) and ALVAC 4 (closed circle). Horizontal bars show medians. The dotted line represents the positive limit of detection for intracellular cytokine staining. (b) Representative analysis of HIV-specific CD4$^+$ T-cell polyfunctionality. Following stimulation with peptide pools, PBMCs were stained simultaneously for CD40L, IFN-γ, TNF-α, IL-2, and MIP-1β and analyzed using nine color flow cytometry. (c) The pie charts depict the background adjusted polyfunctional behavior (1–5 functions) of HIV specific CD4$^+$ T cells.

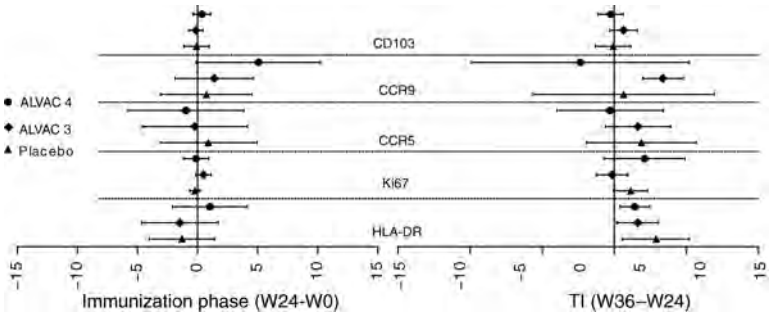


Fig. 4. T-cell activation and tropism. Change from baseline (W24–W0) or from end of immunization during treatment interruption (W36–W24) in the frequencies of total $CD4^+$ T cells expressing markers of activation (HLA-DR, Ki67) and tropism (CCR9, CD103), or the HIV co-receptor CCR5.

significant change was observed following vaccination, a slight increase in CCR5, the HIV-co-receptor, was observed on $CD4^+$ T cells, coincident with treatment interruption (Fig. 4). Furthermore, the expression of activation markers, and in particular HLA-DR, was increased after treatment interruption (but not vaccination) on $CD4^+$ T cells, although we did not observe any significant differences between the groups (Fig. 4).

ALVAC-HIV immunization was not associated with the induction of HIV-specific enhancing antibodies

Given the rapid depletion of HIV-specific $CD4^+$ T cells in the vaccine arms, the possibility that immunization may have also induced antibodies that could enhance HIV infection in target cells was next explored. Of the sera tested, only two sera collected at W28 contained neutralizing antibodies, exhibiting 90% neutralizing activity of either the BIG strain or the KON strain at a 1 : 10 or 1 : 20 dilutions, respectively. However, none of the sera contained enhancing antibodies.

Discussion

Instead of protection, therapeutic vaccination using vCP1452 led to enhanced viral replication following treatment interruption and more rapid kinetics to therapy resumption in patients receiving more doses of the vaccine. We therefore performed extensive immunological follow-up, aimed at defining the potential correlates of enhanced viral rebound in vaccinees. The results strongly suggest that the ALVAC-HIV was only weakly immunogenic, failing to elicit significant HIV-specific $CD8^+$ T-cell responses, thought to be critical for antiviral control, but rather induced mainly activated $CD4^+$ T-cell responses that were rapidly depleted following viral rebound at treatment interruption.

To define whether biases existed among the 3-vaccine, 4-vaccine, or placebo group, we compared several clinical parameters to determine whether any particular characteristic was associated with this differential outcome following treatment interruption. However, no differences were observed among the groups for CD4 nadir prior to ART, as has been previously described [8]. Similarly, no differences in viral load rebound or timing to ART resumption were associated with the distribution of protective or nonprotective HLA alleles among the three arms. Furthermore, virologic analyses also suggested that no difference was observed in viral tropism following immunization or discontinuation of therapy in vaccinees that could have impacted on viral dissemination following treatment interruption (data not shown). These data strongly suggest that neither disease progression markers, HLA alleles, nor viral tropism accounted for differential responses to immunization among the vaccine and placebo groups.

We next hypothesized that enhanced viral replication among the vaccinees, may have been due to immunization induced T-cell activation that could result in the generation of enhanced frequencies of target cells for viral replication following treatment interruption. Detailed analysis of T-cell immune activation markers, however, failed to show significant differences after 3-vaccine or 4-vaccine injections, despite changes in activation or tropism markers, arguing that enhanced viral replication was not associated with vaccine-induced systemic immune activation. Thus these results are in line with previous studies reporting an enhancement of viremia despite a lack of systemic activation following vaccination [10,29]. Nevertheless we cannot exclude that local immune activation, particularly in tissues or lymph nodes where the virus replicates most profusely, might occur following vaccination that could enhance the frequency of locally activated target cells that could fuel viral replication/production.

Therefore, we speculated that this deleterious effect of vaccination may have been due to weak vaccine immunogenicity and a skewed induction of potential HIV-specific target $CD4^+$ T cells. In fact, two experimental techniques, including intracellular cytokine staining after peptide-pool stimulation and tetramer analysis of HIV-specific $CD8^+$ T-cell frequencies demonstrated a nonsignificant increase in CTL numbers,

their activation, or their ability to produce cytokines during the immunization period. Such a modest induction of HIV-specific $CD8^+$ T-cell responses did not appear to reflect an overall HIV-associated immune-anergy as HIV-specific $CD8^+$ T-cell numbers and activation increased in the same patients after virus re-exposure. Rather this lack of HIV-specific $CD8^+$ T-cell responses was more reflective of weak vaccine immunogenicity, although the same construct was able to successfully induce HIV-specific $CD8^+$ T-cell responses in patients with healthier immune status, such as those in primary HIV infection [12]. Instead, vaccination resulted in a preferential expansion of HIV-specific $CD4^+$ T-cell responses, although to a modest level, similar to what has been recently reported in the preventive RV144 vaccine trial performed in uninfected patients [13]. The results indicate that this immunogen tends to skew the HIV-immune response towards $CD4^+$ T cells, as had been previously shown in healthy volunteers [14,30,31], reflecting a potential characteristic of poxvirus immunogenicity [32].

Finally, vCP constructs typically poorly induce humoral immunity [14]. Similarly, low neutralizing antibody titers were observed. Furthermore, no evidence of enhancing antibodies was observed following vaccination that could account for increased viral replication among vaccine recipients.

The present immunological study enables us to propose several mechanisms that could underlie the higher viral rebound and more rapid kinetics of ART resumption in patients receiving the ALVAC-HIV vaccine compared to placebo controls. While the vaccine did not modulate the HIV-specific $CD8^+$ T cells, HIV viral rebound profoundly activated and induced the expansion of $CD8^+$ T cells following treatment interruption. On the other hand, four doses of the ALVAC-HIV vaccine enhanced the frequency and boosted the polyfunctional capacity of HIV-specific $CD4^+$ T cells in ART-treated HIV-infected patients. Thus the vaccine resulted in an activation of HIV-specific $CD4^+$ T-cell response potentially representing an immunization-induced niche within which the virus may have been able to replicate more proficiently following treatment interruption. Moreover, due to the deficit in vaccine-induced stimulation of antiviral HIV-specific $CD8^+$ T-cell responses, the present immunization procedure may have induced target cells for viral replication in the absence of the cytolytic effector cells that could contain viral replication. Thus, while $CD4^+$ T-cell help has been shown to be critical for the induction of functional $CD8^+$ T-cell responses [33], the generation of $CD4^+$ T-cell responses in the absence of effector T-cell activity may be deleterious to vaccinees.

Overall these results suggest that ALVAC-HIV therapeutic immunization, with its limited HIV-specific $CD8^+$ T-cell responses, is not able to induce virus control and can potentially adversely render patients more susceptible to disease progression upon skewed induction of activated $CD4^+$ T-cell targets. Thus, therapeutic vaccine strategies that can induce robust and broad $CD8^+$ T-cell responses, while only modestly activating $CD4^+$ T-cell responses, may provide a more effective strategy to induce viral control.

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L.P., G.A., A.G.M., M.A., C.K., and B.A. designed the research. L.P., G.A., and M.B. performed the experiments. L.P. collected and analyzed the data. R.M., F.G., B.C., and C.K. provided patients' samples. M.L. provided reagents and helped with the experimental design. L.A. and D.C. performed the statistical analyses. L.P., B.A., and G.A. wrote the paper.

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