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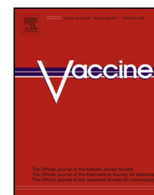

Boosters of a therapeutic HIV-1 vaccine induce divergent T cell responses related to regulatory mechanisms☆,☆☆

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ABSTRACT

Therapeutic human immunodeficiency virus (HIV) vaccines aim to reduce disease progression by inducing HIV-specific T cells. Vacc-4x are peptides derived from conserved domains within HIV-1 p24 Gag. Previously, Vacc-4x induced T cell responses in 90% of patients which were associated with reduced viral loads. Here we evaluate the effects of Vacc-4x boosters on T cell immunity and immune regulation seven years after primary immunization. Twenty-five patients on effective antiretroviral therapy received two Vacc-4x doses four weeks apart and were followed for 16 weeks. Vacc-4x T cell responses were measured by proliferation (CFSE), INF-γ, CD107a, Granzyme B, Delayed-Type Hypersensitivity test (DTH) and cytokines and chemokines (Luminex). Functional regulation of Vacc-4x-specific T cell proliferation was estimated *in vitro* using anti-IL-10 and anti-TGF-ß monoclonal antibodies.

Vacc-4x-specific $CD8^+$ T cell proliferation increased in 80% after either the first (64%) or second (16%) booster. Only 40% remained responders after two boosters with permanently increased Vacc-4x-specific proliferative responses ($p = 0.005$) and improved $CD8^+$ T cell degranulation, IFN-γ production and DTH. At baseline, responders had higher $CD8^+$ T cell degranulation ($p = 0.05$) and $CD4^+$ INF-γ production ($p = 0.01$), whereas non-responders had higher production of proinflammatory TNF-α, IL-1α and IL-1ß ($p < 0.045$) and regulatory IL-10 ($p = 0.07$).

Notably, IL-10 and TGF-ß mediated downregulation of Vacc-4x-specific $CD8^+$ T cell proliferation increased only in non-responders ($p < 0.001$). Downregulation during the study correlated to higher PD-1 expression on Vacc-4x-specific $CD8^+$ T cells ($r = 0.44$, $p = 0.037$), but was inversely correlated to changes in Vacc4x-specific $CD8^+$ T cell proliferation ($r = -0.52$, $p = 0.012$).

These findings show that Vacc-4x boosters can improve T cell responses in selected patients, but also induce vaccine-specific downregulation of T cell responses in others. Broad surveillance of T cell functions during immunization may help to individualize boosting, where assessment of vaccine-related immune regulation should be further explored as a potential new parameter.

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1. Introduction

Human immunodeficiency virus (HIV) type 1 preferentially targets and depletes two types of activated $CD4^+$ T cells in primary infection; $CCR5^+CD4^+$ Th17 mucosal T cells [1] and subsequently HIV-specific $CD4^+$ T cells. The latter clones are essential in maintaining effective viral control in collaboration with $CD8^+$ T cells of the same specificities [2,3]. In chronic HIV infection there is an altered balance between immune activation, immune exhaustion and regulation. Recent data suggests that low T regulatory cell (Treg) responses are associated with viral control in HIV controllers

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[4] and that protective HIV-specific CD8+ T cells evade Treg suppression [5].

Therapeutic HIV vaccines aim to repair HIV-specific cellular immunity after primary infection by inducing new T cell clones and thereby better control viral replication, especially through polyfunctional HIV-specific CD8+ T cell responses [6,7]. Thus, effective vaccination may potentially reduce disease progression, delay initiation of antiretroviral treatment (ART) and help to decrease residual viral load levels in already treated patients [8].

Vacc-4x is a therapeutic HIV-1 vaccine candidate derived from conserved domains within p24 Gag, designed to stimulate cellular immune responses [9]. Previously, Vacc-4x stimulated proliferative T cell responses in 90% of immunized HIV positive patients [10]. Enhanced T cell responses to Vacc-4x were associated with reduced viral loads during ART interruptions [11,12] and postponed reintroduction of ART [10] without inducing viral immune escape [13]. Furthermore, we have demonstrated long-term Vacc-4x-specific T cell memory in the same cohort [9,14].

The objective of this study was to evaluate how two booster immunizations of Vacc-4x would influence quantitative and qualitative aspects of Vacc-4x-specific T cell immune responses in previously immunized subjects, with the ultimate goal to explore the potential of developing individualized immunization regimen for HIV-infected patients. Although vaccine boosters aim to strengthen the pool of effector T cells, boosting might theoretically also induce or enhance a spectra of immune regulatory mechanisms [15,16]. However, to our knowledge, regulation has not been assessed during therapeutic HIV vaccine boosters in man. We hypothesized that responses to two consecutive boosters would be differentiated and that immune regulatory mechanisms might play a role. We assessed the regulation of vaccine-specific T cell proliferation by the two key inhibitory soluble cytokines IL-10 and TGF-ß *in vitro* [16,17]. Instead of characterizing the many possible cellular sources for these cytokines, we here examined their collective influence on T cell effector function in the individual patient. We found that changes in this parameter could explain the diversified responses to booster vaccination.

2. Materials and methods

2.1. Study participants and study design

Twenty-five HIV positive patients who had completed the first Vacc-4x study and who had been on effective ART for at least 6 months, with viral load <20 copies/ml and with no clinical signs of immune deficiency were included for re-immunization in this non-randomized single-center, open-label one arm study. Patients with previous allergic reactions to Vacc-4x, malignant disease, immune suppressive therapy, concurrent active infections, as well as pregnant or breastfeeding women were excluded. Fifteen minutes prior to immunizations, low dose GM-CSF (Leukine; Genzyme, MA, USA) was injected intradermally followed by 100 μl Vacc-4x (300 μg/ml per peptide) intradermally in the same spot at baseline and after 4 weeks. Clinical examination, general biochemistry and hematologic analyses, HIV-RNA, CD4 and CD8 cell counts were performed on every visit at study weeks 0, 4 and 16. All blood samples were collected prior to immunizations.

The study was approved by the Norwegian Medicine Agency and the Norwegian South-Eastern Regional Committee for Medical and Health Research Ethics with written informed consent from all participants and monitored by Mericon (Skien, Norway).

2.2. T cell assays

2.2.1. Proliferation and IL-10 and TGF-β blocking assays

Peripheral-blood mononuclear cells (PBMC) were labelled with carboxyfluorescein succinimidyl ester (CFSE, Invitrogen Molecular Probes, OR, USA) as described earlier [9] before stimulation with Vacc-4x (5 μg/ml/peptide) along with unstimulated negative and positive controls (Staphylococcal enterotoxin B, 0.5 μg/ml, Sigma–Aldrich, MO, USA). In addition, parallel antigen-stimulated samples and controls cultures received blocking anti-IL-10 and anti-TGF-ß monoclonal antibodies (mAbs) at 10 μg/ml, according to the instructions by the manufacturer (R&D Systems Europe, Abingdon, UK) and described elsewhere [17]. Cells were cultured in serum-free culture medium (Gibco AIM V, Invitrogen) with 0.5% highly purified human albumin for 6 days at 37 °C and 5% CO_2 before harvesting, staining and preparation for flow cytometric analysis as previously detailed [9]. Cells were stained with anti-CD3 Pacific Blue, anti-CD8 AmCyan, anti-HLA-DR PE-Cy7 and 7-aminoactinomycin (7-AAD), the latter to exclude nonviable cells (Becton Dickinson Pharmingen, NJ, USA). Antigen-specific response was calculated as difference in percentage of proliferated (CFSE^{dim}), activated (HLA-DR$^+$) and live (7-AAD$^-$) CD3$^+$ T cell subsets (CD8$^+$ or CD8$^-$ (defined as CD4$^+$), respectively) between antigen-stimulated and control cultures, as previously detailed [14]. Multiple parallels were not used to shortage of available cells. IL-10- and TGF-β-mediated regulation of proliferation was estimated by subtracting antigen-induced proliferation from parallel controls with only IL-10- and TGF-β blocking mAbs (Fig. 1). Flow cytometry data were obtained with a BD Canto II with BD Diva software v6 and analyzed in WinList v7 (Verity Software House, ME, USA).

2.2.2. INF-γ and CD107a degranulation assays

Freshly isolated PBMC were stimulated with Vacc-4x (5 μg/ml/peptide) at 37 °C and 5% CO_2 for 6 h followed by harvesting, staining and preparation for flow cytometric analysis. Monensin (BD) and FITC-labelled anti-CD107a (eBioscience, CA, USA) were added prior to stimulation [18]. Two-step surface staining was performed with biotinylated anti-PD-1 (R&D) and Streptavidin-APC (Invitrogen), the latter with anti-CD3 and anti-CD8, followed by permeabilization (PERMII, BD) and intracellular staining for interferon-γ (INF-γ) PE (BD). Cut-off for PD-1 was determined using the Fluorescence minus one method [19].

2.2.3. Granzyme B ELISPOT

The Granzyme B enzyme-linked immunosorbent spot (ELISPOT) assay was performed according to the instructions by the manufacturer (Mabtech, Sweden) with cryopreserved PBMC that were reconstituted overnight and stimulated in triplicate with Vacc-4x 15-mer overlapping peptides along with positive and negative controls using 200,000 PBMC/well. Spots were counted using an AID Elispot reader with AID Elispot v5 scanner software (AID GmbH, Germany) and median values of triplicates were used for analysis. Spot-forming units (SFU) were adjusted by the number of spots in negative controls. The proportion of CD8+ T cells in each sample was enumerated by flow cytometry to calculate SFU per million CD8+ T cells.

2.2.4. Soluble cytokine and chemokine assay

Cytokines and chemokines were measured in supernatants from cell cultures containing 200,000 PBMC/well from the proliferation assay after 24 h stimulation with Vacc-4x. IL-1β, IL-8, IL-10, IL-13, IL-17, IFN-γ-induced protein 10 (IP-10), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1β (MIP-1β), RANTES and tumor necrosis factor-α (TNF-α) were measured using the BioPlex XMap technology (TX, USA) with a Luminex IS100 instrument (BIO-RAD, CA, USA) and Bio-Plex manager Software v6.

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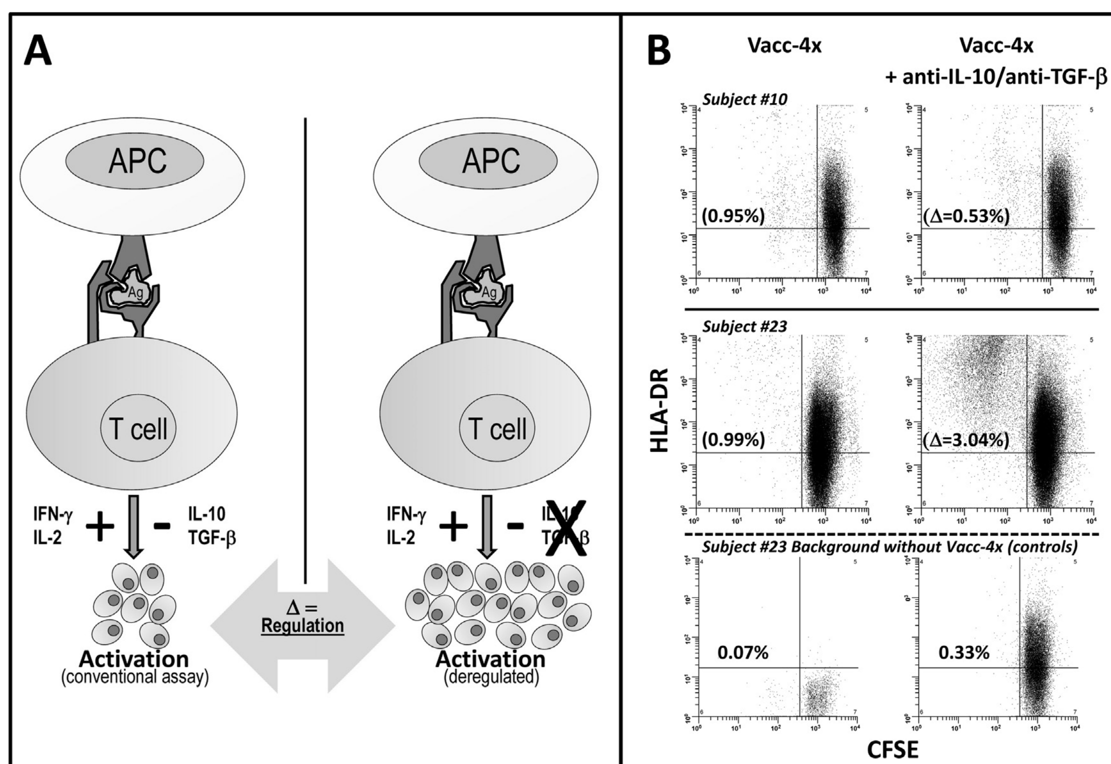

Fig. 1. (A) Schematic outline of the assay for assessing T cell regulation of vaccine-specific proliferation. Left panel illustrate that antigen-induced T cell responses, such as proliferation, is a net result of proinflammatory and regulatory signals. Functional IL-10 and TGF-β-mediated T cell regulation (*Δ*) is defined here as the difference in conventional antigen-induced proliferation (left panel) *versus* antigen-induced proliferation in the presence of anti-IL-10 and anti-TGF-ß monoclonal antibodies (right panel). (B) Example of two study patients with similar conventional proliferative CD8$^+$ T cell responses (CFSE^{dim}HLA-DR$^+$, left panels) to Vacc-4x peptides (0.95% and 0.99%, respectively) above corresponding unstimulated control. Parallel cultures with additional anti-IL-10 and anti-TGF-β (right panels) reveal moderately increased (*Δ* = +0.53%, subject #10, upper right panel) or strongly increased (*Δ* = +3.04%, subject #23, lower right middle panel) proliferation above those with Vacc-4x alone after correcting for Vacc-4x unstimulated control with anti-IL-10 and anti-TGF-β only, Δ being denoted as downregulation of CD8$^+$ T cells. The two lower panels shown controls without Vacc-4x peptides for subject #23 in the absence (left lower panel) or presence (right lower panel) of anti-IL-10 and anti-TGF-β blocking mAbs, respectively.

The StatLIA software package v3 (Brendan Scientific Inc., CA, USA) was used to calculate sample cytokine concentrations.

2.2.5. Delayed-type hypersensitivity testing (DTH)

Vacc-4x peptides were injected intradermally without GM-CSF at study end. The perpendicular diameters of palpable skin infiltrates were registered after 48 h as previously described [11,20].

2.3. Statistics

Responders and non-responders after the first (“primary”) or second (“overall”) booster were defined by positive or negative differences relative to baseline in vaccine peptide-specific CD8$^+$ T cell responses, respectively. Mann–Whitney U (MWU), Kruskal–Wallis test, Spearman Rank, Wilcoxon signed-rank test and Fisher exact test were used to analyze differences between groups, correlations, dependent variables and cross-tabulated data, respectively (Statistica v7, StatSoft, OK, USA). Continuous variables are presented as median (interquartile ranges, IQR). A p-value $\leq$0.05 was considered significant.

3. Results

3.1. Safety and clinical data

Twenty-five of the 38 patients from the first Vacc-4x study [9] were available and eligible for re-immunization. After primary immunizations, all continued the 14 weeks per protocol interruption of ART for 1.8 years (median) and were thereafter treated with effective ART for 5.4 years (median) before inclusion in the present study. Clinical data are presented in Table 1.

After two Vacc-4x booster doses, only mild and transient discomfort was reported; 15 patients developed moderate local erythema or swelling at the injection site and five experienced additional muscle pain, fever, fatigue and headache after the first booster, whereas 18 patients reported local and three patients reported systemic symptoms after the second booster. No HIV-related complications were seen during the study period and all participants were asymptomatic at study end. Moreover, no changes were noted for median CD4 cell counts (587 *vs.* 582 cells/μl), CD8 cell counts (1227 *vs.* 1151 cells/μl) or HIV-RNA (<20 copies/ml).

3.2. Various patterns in Vacc-4x induced T cell responses following booster immunizations

Vacc-4x-specific *in vitro* responses were measured by T cell proliferation, CD8$^+$ T cell degranulation (CD107a and Granzyme B) and INF-γ production with rather large variations (Fig. 2). However, the data were consistent throughout the study with significant correlations between the CD8$^+$ and CD4$^+$ T cell subsets as well as for the parameters for degranulation (data not shown).

Robust proliferative CD8$^+$ T cell responses to Gag have consistently been related to slow progression of HIV [7,20]. Change in Vacc-4x proliferative CD8$^+$ T cell responses relative to baseline was therefore chosen as the primary immunological read-out for boosting efficacy, in accordance with previous Vacc-4x studies [9].

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Table 1
Clinical data at inclusion.

	All ($n=25$)		Overall responders ($n=10$)		Overall nonresponders ($n=15$)	
Age (years)	51	(48–56)	52	(49–56)	50	(47–58)
DTH (mm^2)[a]	79	(39–154)	143[b]	(104–398)	51	(28–133)
Years since HIV diagnosis	14.7	(13.0–19.4)	15.6	(12.2–20.9)	14.6	(13.6–19.4)
Years on effective ART	5.4	(3.5–6.1)	5.4	(3.3–6.1)	4.8	(3.8–6.1)
Nadir CD4 (cells/μl)	210	(150–230)	205	(150–230)	210	(150–270)
CD4 count (cells/μl)	587	(435–815)	702	(435–873)	566	(423–687)
CD8 count (cells/μl)	1227	(956–1571)	1155	(813–1553)	1233	(1026–1780)
HIV RNA (copies/ml)	<20	(<20–<20)	<20	(<20–<20)	<20	(<20–<20)

Data presented as median (interquartile range).
[a] DTH: delayed-type hypersensitive skin test (induration area) at week 200 of the initial Vacc-4x protocol [10,11].
[b] $p=0.056$ between overall responders and non-responders (Mann–Whitney U test).

At baseline, 19 (76%) patients had detectable proliferative CD8+ T cell responses to Vacc-4x (0.90% [0.28–1.32]). Twenty patients (80%) enhanced Vacc-4x CD8+ proliferation at some time point, either after the first (64%) or the second booster (16%). At the end of the study, 10 patients (40%) ended up as *overall responders* with permanently improved Vacc-4x CD8+ T cell proliferation relative to baseline ($p=0.005$). The remaining 15 patients (60%) were *overall non-responders*, notably with decreasing Vacc-4x CD8+ T cell proliferation after two boosters ($p=0.001$), with the same distribution of randomized Vacc-4x dose arms as overall responders (see [9]). However, quite different responses after the *first* booster were observed within each responder group (Fig. 3, right panels): for example, among *overall responding* patients, 5 patients achieved maximal proliferative responses after the first booster, but with a four-fold reduction in proliferation after the second ($p=0.046$). In contrast, the 4 out of the 5 remaining *overall responders* tended to reduce responses after the first booster. Among the overall non-responders, 10 patients improved Vacc-4x responses after the first booster whereas proliferation decreased in 5 (Fig. 3). Thus, across the overall response groups, a subgroup of patients seemed to profit from the first booster only, denoting them as *primary responders*, with the reservation that first and second responses were evaluated after different period of times relative to boosting.

At baseline, *overall responders* had higher frequencies of Vacc-4x-specific CD8+ T cell CD107a+ degranulation ($p=0.05$) and CD4+ T cell INF-γ production ($p=0.01$), whereas some *overall non-responding* patients had higher secretion of certain proinflammatory cytokines, but also IL-10 (see below). In addition,

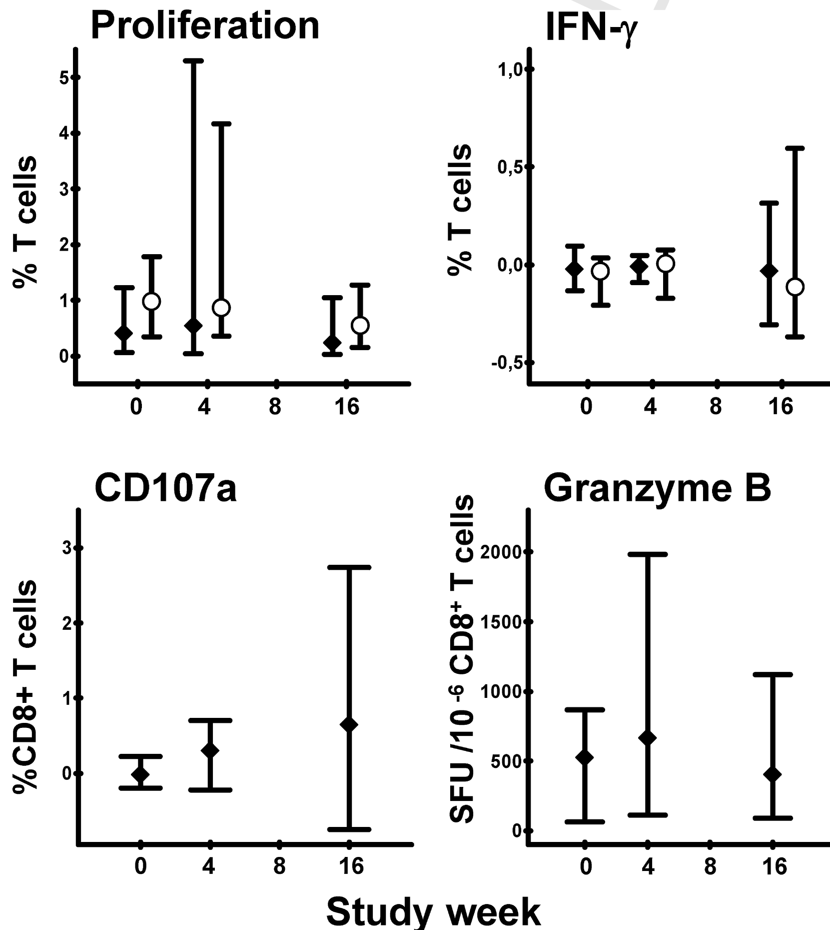

Fig. 2. Vacc-4x-specific T cell responses at baseline (week 0), after the first (week 4) and second booster (week 16), measured by four different *in vitro* assays ($CFSE^{dim}$HLA-DR+-defined proliferation, CD107a, Granzyme B and INF-γ). Medians and interquartile range (IQR) indicated for CD8+ (♦) and CD4+ (○) T cells, respectively.

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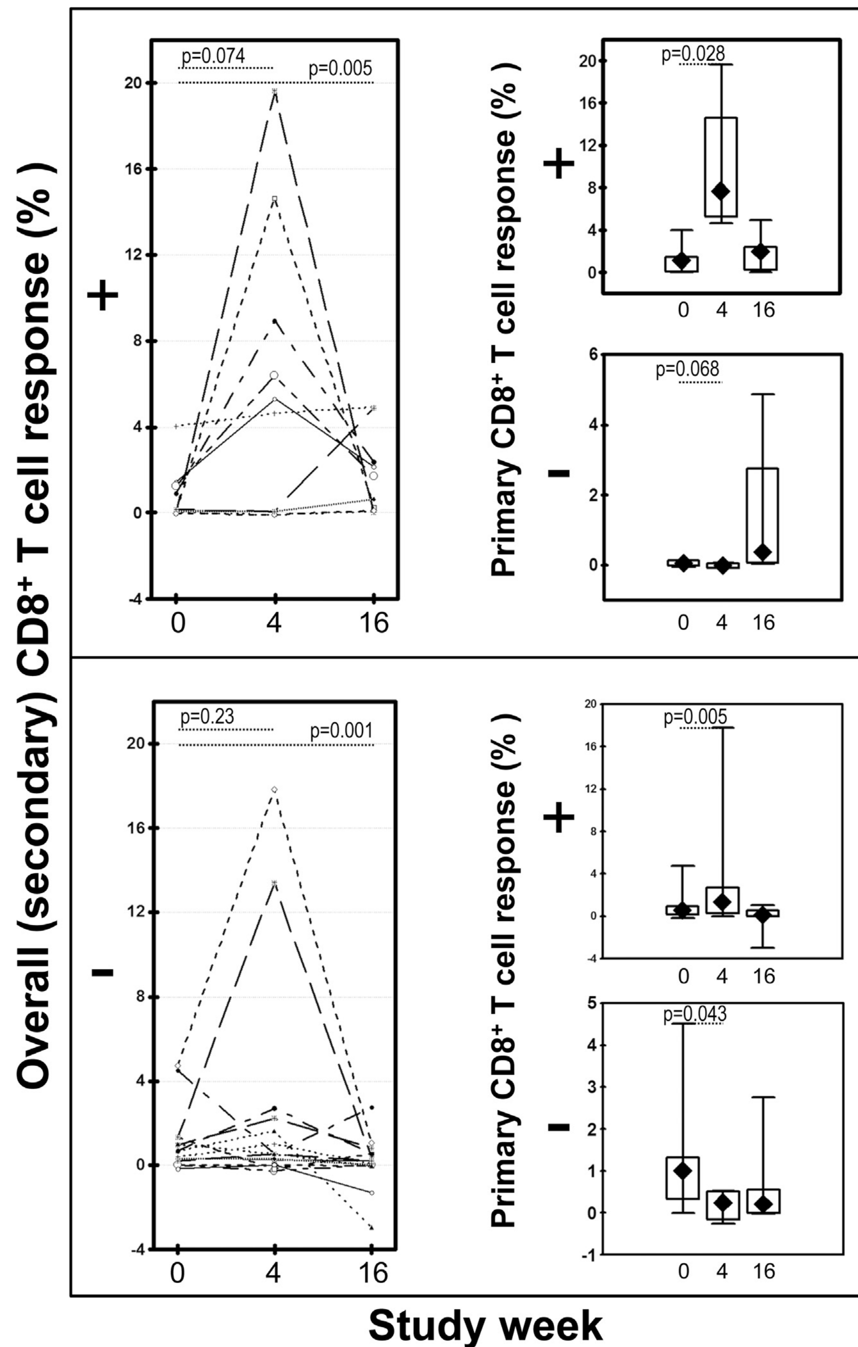

Fig. 3. Time-dependent changes in proliferative CD8+ T cell responses to Vacc-4x peptide panels. Patient case profiles illustrated for overall responders at end of study (+, upper left panel) and overall non-responders (−, lower left panel), respectively. Smaller box-plot panels show these responder groups according to their primary Vacc-4x response after the first booster determined at week 4 (medians, interquartile ranges and overall range indicated). *p*-values for groupwise differences relative to baseline (dashed horizontal lines) are shown (Wilcoxon).

primary responders had higher baseline levels of Vacc-4x-specific degranulating (CD107a+) CD8+ T cells ($p = 0.05$, data not shown). No differences in baseline proliferative response rates (70% and 80%, $p = 0.46$), clinical parameters, HIV-RNA levels or CD4 cell counts were seen between *overall* responders and non-responders (Table 1).

Additional modalities of Vacc-4x induced CD8+ T cell responses after two boosters were also assessed and evaluated together. Nine (90%) overall responders improved at least two CD8+ T cell response modalities (mainly degranulation) and 40% of them even had improvements of more than two, which tended to be lower for *overall non-responders* (55% and 7%, respectively; $p < 0.07$, Fisher).

3.3. *Higher baseline levels of proinflammatory soluble factors in Vacc-4x overall non-responding patients*

Cytokine and chemokine levels were measured in cell culture supernatants at weeks 0 and 16. At baseline, the overall proliferative CD8+ T cell response correlated both with Th1 (TNF-α; $r = 0.55$, $p = 0.012$) and Th2 cytokines (IL-13; $r = 0.51$, $p = 0.02$). Interestingly, the 5 overall non-responders who lost proliferative responses already after the first booster, had in fact the highest baseline levels of the proinflammatory factors TNF-α, IL-1b, MIP-1b and possibly also inhibitory IL-10 in response to *in vitro* stimulation with Vacc-4x peptides (Fig. 4). After two booster immunizations, overall

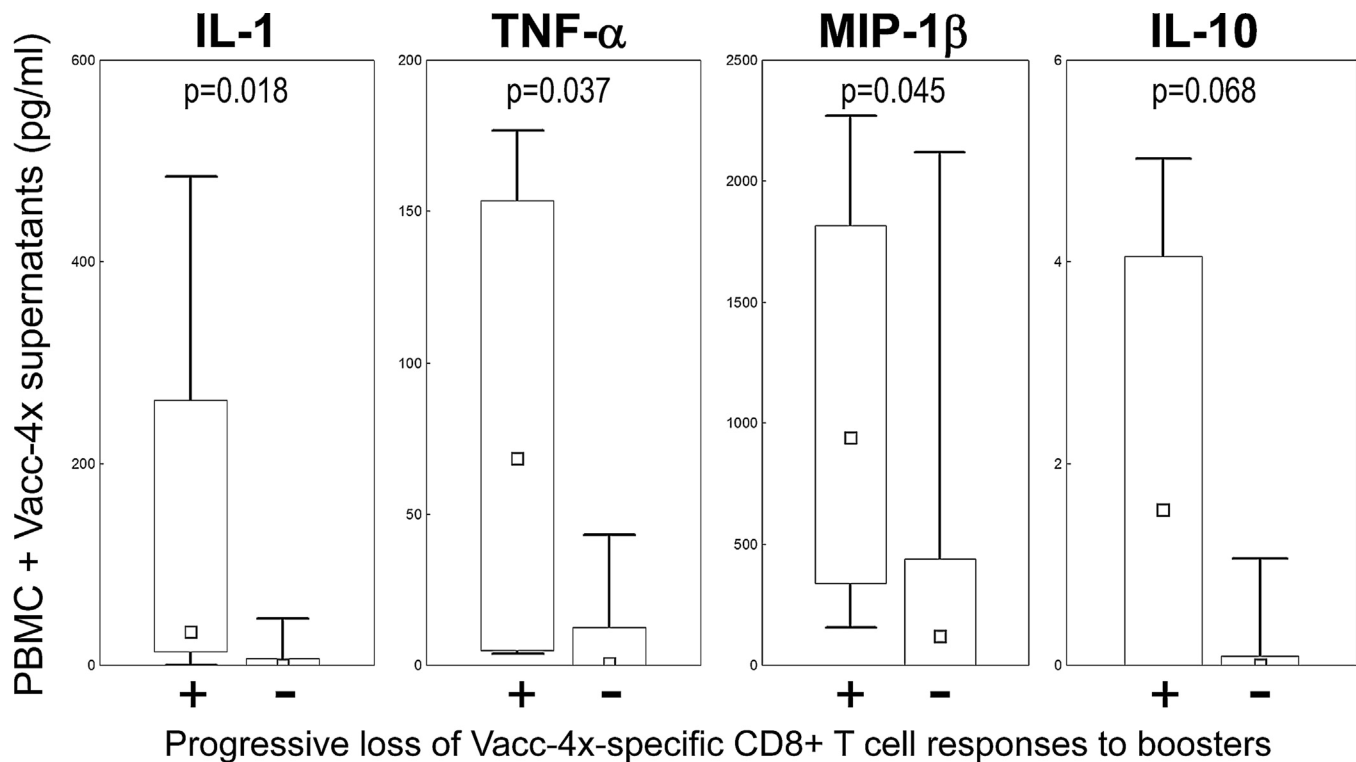

Fig. 4. Supernatant concentrations after stimulation of PBMC with Vacc-4x peptides at study baseline, adjusted for levels in control cultures. Proinflammatory cytokines (IL-1, TNF-α, and TGF-β) and inhibitory IL-10 shown for patients with progressive loss of CD8+ T cell responses after each of the two booster (+, $n=5$) are compared with the other participants (−, $n=20$), *p*-values indicated. Data are expressed as medians, IQR and range.

non-responders increased Vacc-4x induced secretion of the Th2 cytokine IL-13 ($p=0.003$, Wilcoxon) whereas overall responders increased secretion of MCP-1 ($p=0.009$). Taken together, at baseline proinflammatory mediators, but also IL-10, were preferentially produced in those patients who did not profit on re-immunizations at all, while boosters particularly enhanced Vacc-4x-related production of the Th2 cytokine IL-13 in the same patients.

3.4. Delayed type hypersensitivity responses to Vacc-4x in vivo

Multiple DTHs were given during the initial Vacc-4x protocol [9] and in follow up studies. Most patients were anergic before primary immunization and developed maximal induration at study week 3 [10,11]. The last DTH before reboost was done at study week 200 of the initial Vacc-4x protocol, approximately three years before the current study. A total of 82% of the reboost patients demonstrated positive DTH >10 mm^2 at week 200 (79 mm^2 [39–154]) with possibly larger DTH in the overall responding reboost patients (Table 1).

In the present study, Vacc-4x DTH was again tested at end of study week 16, twelve weeks after the second booster. Twenty-two (96%) of the 23 patients tested had positive DTH (99 mm^2 [64–177]), still with possibly larger indurations in overall responders than non-responders ($p=0.056$). The relevance of this simple *in vivo* test was supported by correlations with the last preceding Vacc-4x DTH ($r=0.57$, $p=0.011$), current $CD8^+$ T cell proliferation ($r=0.56$, $p=0.006$) and increased production of IL-13 ($r=0.63$, $p=0.003$).

3.5. Increased IL-10 and TGF-β mediated downregulation of Vacc-4x T cell proliferation in overall non-responding patients

We next assessed the association between boosting, T cell proliferation and T cell regulation mediated by the key regulatory cytokines IL-10 and TGF-β as illustrated in Fig. 1. At baseline, *in vitro* downregulation of Vacc-4x-specific proliferation was similar in magnitude and prevalence between the $CD8^+$ and $CD4^+$ T cell subsets (16 [67%] and 15 [63%]) and between overall responders and non-responders. Notably, this parameter changed only in overall non-responders, who downregulated Vacc-4x-specific $CD8^+$ T cell proliferation ($p<0.001$, Wilcoxon). Consequently, overall non-responders demonstrated stronger *in vitro* IL-10 and TGF-ß mediated downregulation of Vacc-4x-specific $CD8^+$ T cell proliferation than overall responders after two boosters ($p=0.031$).

The finding that two boosters either enhanced proliferation or induced downregulation of Vacc-4x proliferation was supported by an overall negative correlation between these two parameters (Fig. 5). Another inhibitory mechanism of effector T cells is expression of PD-1 [21,22]. Changes in the downregulation of Vacc-4x proliferation correlated positively to concurrent changes in the expression of PD-1 on Vacc-4x-specific $CD8^+CD107a^+$ T cells (Fig. 5).

4. Discussion

The objective of this study was to evaluate the effects of two Vacc-4x boosters on specific T cell immunity in previously immunized HIV-infected subjects. Based on the substantial diversity in HIV-specific responses in general [17,23] and the variability we have seen in Vacc-4x responses [10], we expected differentiated responses to boosters and hypothesized a possible link to immune regulation.

In this cohort, most patients still had detectable Vacc-4x memory responses more than seven years after the primary immunization. The overall efficacy of two boosters was only 40% in terms of proliferative $CD8^+$ T cell responses. However, 80% of the patients were responders at some time point, after either one or two boosters. Notably, only one patient was identified who responded positively to the first and then again to the second booster, and 20% progressively lost Vacc-4x responsiveness after each booster.

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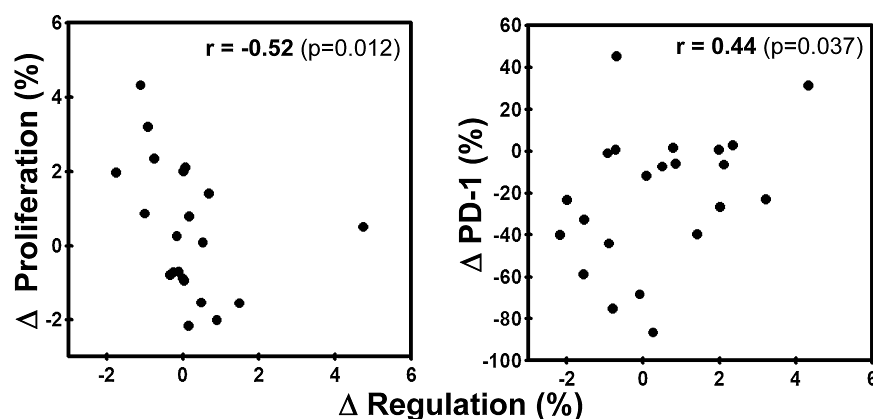

Fig. 5. Changes from baseline to end of study after two boosters in Vacc-4x induced CD8+ T cell regulation by IL-10 and TGF-β (*x*-axis) and inverse correlation with corresponding changes (*y*-axis) in proliferative CD8+ T cell responses (left panel) and positively so with PD-1 expression on CD8+CD107a+ T cells (right panel). Spearman rank correlations and *p*-values indicated.

Overall responders did not only improve CD8+ proliferation but also degranulative capacity, which is an important characteristic of effective cytotoxic effector T cells [24]. Re-immunization may therefore improve several Gag specific cytotoxic CD8+ T cell response modalities in selected patients. Overall responders also demonstrated increased Vacc-4x-specific INF-γ production and CD107a degranulation, suggesting the induction of polyfunctional T cells. Another response parameter was DTH which related to clinical outcome after primary Vacc-4x immunizations [10,19]. In this study, DTH marginally improved compared to the last preceding DTH taken three years earlier and also correlated to CD8+ T cell proliferation.

Nevertheless, most patients (60%) ended up with Vacc-4x-specific proliferation below their baseline levels after two Vacc-4x boosters. To our knowledge, this is the first observation where unsuccessful boosting of a therapeutic HIV vaccine can be explained by increased IL-10 and TGF-ß mediated downregulation of vaccine-specific T cell proliferation, in keeping with facilitation of therapeutic vaccination of mice obtained by IL-10 blockade *in vivo* [25]. Booster-induced changes in IL-10 and TGF-ß mediated regulation were linked to enhanced expression of inhibitory PD-1 on Vacc-4x-specific effector cells. Thus, *in vitro* quantification of T cell regulation might be relevant when selecting patients for vaccine trials as well as for the individualization of booster regimens. For example, our data suggest that some of our current study patients should not have been boosted at all, and robust proliferative responses after the first booster apparently identified patients who in retrospect should not have been offered a second dose. However, further explorative studies should include additional regulatory modalities, such as the functional regulation by PD-1 and CTLA-4 [26].

Several aspects of our study and exploratory variable for regulation need to be addressed. For example, use of cut-off thresholds to define “positive” or non-acceptance of “negative” antigen-specific responses in stimulated samples relative to control might masque regulation as phenomenon. We therefore used the raw data throughout. We acknowledge that classification of responder based on minute differences in responses relative to baseline in some patients may be different by chance and altogether weaken the statistical analysis, but such cases should be randomly distributed. Moreover, the limited cohort size calls for confirmatory trials, but we were not able to recruit all previously vaccinated participants. In addition, regulation and activation might develop differently by comparing different peptide booster doses, which was fixed in this study, or with individualized time intervals between boosters. Since the Th2 cytokine IL-13 increased, it would also have been interesting to test antibody levels to the Vacc-4x peptides, which have been negligible in previous studies. It is also possible that booster-induced T cell regulation may develop differently with other vaccines or modes of immunization. Ideally, we also would have wanted some additional data: Firstly, future trials of therapeutic HIV vaccines with patients on ART should strive for ultrasensitive HIV RNA. However, this was here hampered mainly by insufficient plasma volume both from the current trial and previous follow-up of the cohort as well as lack of facilities in our region for this test. Secondly, characterization of the cellular sources of IL-10 and TGF-β in each individual patient might have gained additional relevant information, although the combined actions of regulatory cytokines on T cell effector functions were in our opinion the most important outcome of this pilot study.

Conclusively, these findings show that Vacc-4x boosters variably improves or dampens T cell responses in different patients and that boosting should be individualized. Broad surveillance of T cell functions may help in this respect and assessment of vaccine-related immune regulation should be further explored as a potential new parameter.

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Contributions: Design of study and regulatory issues: DK, MS, BS and IB; acquisition of data and monitoring: AL, KB, IB, JOH and HCA; analysis, interpretation and writing the paper: AL, KB, MS, BS, AMDR, DK

Conflicts of interest: Birger Sørensen is shareholder in Bionor Pharma and Maja Sommerfelt and Jens O. Holmberg were employed by the Company. Otherwise, there are no other conflicts of interest.

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