DNA/NYVAC Vaccine Regimen Induces HIV-Specific CD4 and CD8 T-Cell responses in Intestinal Mucosa

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Abstract

In the present study, we have investigated the anatomic distribution in blood and gut mucosal tissues of memory poxvirus-specific CD4 and CD8 T cells in subjects vaccinated with smallpox and compared with vector (NYVAC)-specific and HIV insert-specific T-cell responses induced by an experimental DNA-C/NYVAC-C vaccine regimen. Smallpox-specific CD4 T-cell responses were present in the blood of 52% of subjects studied, while Smallpox-specific CD8 T cells were rarely detected (12%). With one exception, Smallpox-specific T cells were not measurable in gut tissues. Interestingly, NYVAC vector-specific and HIV-specific CD4 and CD8 T-cell responses were detected in almost 100% of the subjects immunized with DNA-C/NYVAC-C in blood and gut tissues. The large majority (83%) of NYVAC-specific CD4 T cells expressed α4β7 integrins and the HIV co-receptor CCR5. These results demonstrate that the experimental DNA-C/NYVAC-C HIV vaccine regimen induces the homing of potentially protective HIV-specific CD4 and CD8 T cells in the gut, the port of entry of HIV and one of the major sites for HIV spreading and depletion of CD4 T cells.
Introduction

Replication-defective adenovirus (Ad) and poxvirus-derived vectors are among the most studied T-cell based vaccine platforms against the Human Immuno-deficiency Virus (HIV) (23, 38).

Recently, two HIV vaccine trials evaluating the efficacy of these two vectors generated different clinical outcomes. The phase IIb test-of-concept efficacy study called STEP which evaluated a trivalent Ad5-Gag/Pol/Nef vaccine candidate was prematurely terminated due to the lack of efficacy of the vaccine (6, 22). Furthermore, unexpectedly the vaccine recipients with higher titers of pre-existing neutralizing antibodies to Ad5 (Ad5 titers >18) and uncircumcised showed increased susceptibility to HIV infection compared to vaccine recipients with lower titers (<18) of Ad5-NAbs and/or circumcised and placebo recipients (6). The increased acquisition of HIV infection in the group of vaccine recipients with higher Ad5-Nabs and uncircumcised has been further confirmed in long-term follow-up analyses (9). These analyses have indicated that the increased susceptibility to HIV infection was concentrated during the 6 months post vaccine administration, suggesting a causal link between Ad5 vaccination, the circumcision status and increased HIV acquisition.

Several hypotheses were proposed to explain the increased acquisition of HIV infection among vaccine recipients of the STEP trial. These include: 1) Ad5 vaccine-mediated activation of Ad5-specific CD4 T cells that in turn may become the ideal targets for HIV infection and supporting virus replication and spreading, 2) generation of ‘enhancing antibodies’ that facilitate HIV infection, 3) activation of dendritic cells (DC) through immune-complexes composed of Ad5 particles and Ad5-NAbs which may facilitate HIV infection of DC and spreading to CD4 T-cells at the site of virus entry, i.e. mucosal surfaces, and 4) unique micro-environment of the mucosal compartment where the mechanisms for increased acquisition to HIV infection likely
operate (8, 33, 37). Despite major efforts, the mechanism(s) responsible for the increased susceptibility to HIV infection in vaccine recipients with higher Ad5-NAb titers remain(s) elusive.

The RV-144 trial evaluated the efficacy of the poxvirus ALVAC-HIV (vCP1521) in combination with a recombinant gp120 subunit vaccine (AIDSVAX B/E) (34). The results indicated that this vaccine combination was effective in preventing infection, i.e. 31.2% efficacy, while it showed no effect on the levels of viremia and/or CD4 T-cell count in vaccinated subjects in whom HIV-1 infection was subsequently diagnosed (34). These results, although showing a modest efficacy, demonstrate for the first time that an HIV vaccine is capable of preventing HIV infection.

HIV vaccine-candidates based on rare adenovirus serotypes such as Ad26 and Ad35 vectors, and poxvirus vectors are important components of promising vaccine candidates for advanced clinical development.

For these reasons, it is important to comprehensively characterize the pre-existing immunity against the virus vector-based vaccines and vaccine-induced immune responses in different anatomical compartments and particularly at mucosal sites which represent the primary port of entry and replication for HIV (4). In the present study, we have characterized the distribution of memory poxvirus and Ad-specific T-cell responses in blood and gut mucosal tissues (rectum and ileum) induced by smallpox vaccination and by adenovirus natural infection in order to evaluate the pre-existing immunity against poxvirus and Ad vector HIV vaccine candidate. Furthermore, poxvirus vector-specific and HIV insert-specific T-cell responses induced by a DNA-C/NYVAC-C (HIV clade C) vaccine regimen were also studied. This vaccine regimen was previously shown (EuroVacc 02 phase I/II clinical trial in HIV uninfected
individuals) to be safe, highly immunogenic (90% of responders) and to induce vigorous,
polyfunctional, broad and durable T-cell responses (13).
Materials and Methods

Study groups. Blood samples and gut biopsies were obtained from thirty HIV-uninfected volunteers and six individuals enrolled in a previous HIV vaccine trial, i.e. EV03 trial (19), who accepted colonoscopy and gut biopsies. The average age in the Smallpox vaccinated group was 52 years and since Smallpox vaccination in Switzerland (Europe) was stopped in 1974, all the subjects in this group received Smallpox vaccination. The average age in the six DNA/NYVAC vaccine group subjects enrolled in the present study was 30 years and only 1 out of 6 subjects (42 years) was Smallpox vaccinated. The individuals enrolled in the EV03 trial were immunized with 3 DNA-C injections (4 mg at week 0, 4 and 8) plus 1 NYVAC-C injection (10^7 pfu at week 24). The six individuals were studied about 2 years after receiving the last immunization. This study was approved by the Institutional Review Board of the Centre Hospitalier Universitaire Vaudois and informed consent was obtained from all volunteers.

Vectors: Two E1/E3-deleted Ad5 vectors (Adβgal and AdGFP) were used in this study and were previously described (32). Briefly, Adβgal harbors a lacZ expression cassette and was used to stimulate Ad-specific T-cells and for in vitro HIV infection (6 x 10^9 pp/ml). AdGFP harbors a GFP expression cassette and was used for the assessment of NAbs titers. Ad5 vector titres were measured in virus physical particles (pp/cell) as described by Mittereder et al. (25). Parental NYVAC and recombinant vector expressing gag, pol and nef of HIV-1 from clade C (referred as NYVAC-C) were previously described (10, 11).

Ad5-NAbs titers: The neutralizing activity of the plasma of each volunteer enrolled was determined by transduction inhibition assays using 911 cell line (30).

Flow cytometry analyses. The following antibodies were used: CD4-APC, CD8-PerCP-Cy5.5, CD3-ECD, CCR5-AF700, beta 7-integrin-PE, alpha-4 integrin-PE-CY5, CLA-biotin, streptavidin-PE-CY7. CD4-APC, CD8-PerCP-Cy5.5 and beta 7-integrin-PE were purchased from
BD Biosciences and CD3-ECD (Beckman Coulter), CLA-biotin, CCR5-AF700 and alpha 4 integrin-PE-CY5 (Biolegend). Dead cells were excluded using the violet LIVE/DEAD stain kit (Invitrogen).

**Ex vivo proliferation assays.** Mononuclear cells were isolated either from peripheral blood using ficoll-histopaque separation (31), or following collagenase digestion of gut biopsies. Five tissue samples were collected in both the terminal ileum and the rectum. Gut samples were taken during routine colonoscopy in patients with normal colonoscopic findings. Colonoscopies were performed under conscious sedation with midazolam and pethidine in moderate doses with a Pentax colonoscope type EC 3890 Fi (Pentax, Tokyo, Japan). Tissue samples were collected with Radial Jaw 4 forceps (Boston Scientific Corporate Natick, MA, USA). Biopsies were then incubated at 37°C for 90 minutes with RPMI medium containing 0.5 mg/ml type II-S collagenase (SIGMA). Mononuclear cells from each anatomic region were re-suspended at 10^6 cells/ml in PBS and incubated for 7 min at 37°C with 0.25 µM 5,6-carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes, USA). The reaction was quenched with one volume of FBS. Subsequently, cells were washed, cultured in 4% human AB serum (Institut Jacques Boy, France) RPMI, and stimulated with ∆E1 Adβgal (10^9 pp/ml), empty-NYVAC (10^6 pfu/ml) or HIV peptide pools encompassing the Gag, Pol, Nef and Env regions (1 µg/ml) for 6 days (1). Cells were also stimulated with Staphylococcus aureus enterotoxin B (SEB) (100 ng/ml) (positive control). At the end of the incubation period, cells were washed and stained with Abs (see flow cytometry section) to CD3, CD4, CD8, CCR5, alpha 4 and beta 7 integrins and the cell-adhesion molecules CLA. Data were acquired on an LSRII four-laser (408, 488, 633 and 405 nm) and analyzed using FlowJo. Between 2 x 10^5 and 10^6 events were acquired in the flow cytometry experiments. The percent of proliferating CD4^+ and CD8^+ T cells, *i.e.* CFSE low cells was
determined from the CD3⁺ cell population. The criteria for scoring as positive the proliferating cell cultures included: a) percent of CFSE low cells >1% after subtracting background (percent of CFSE low cells in unstimulated cell cultures) and b) a stimulation index (SI) >3. The SI was calculated by the fold increase between stimulated versus unstimulated cell cultures. Expression of integrins, chemokine receptors and cell-adhesion molecules on antigen-specific CD4 and CD8 T cells were established on CFSE low proliferating CD4 or CD8 T cells.

**HIV infection in vitro.** Mononuclear cells isolated either from peripheral blood or from gut tissues (rectum + ileum) (n=5) were stimulated with Adβgal (6 x 10⁹ pp/ml) or SEB (positive control) or remained unstimulated. After 3 days of exposure, cell cultures were washed, and exposed to 30 picograms of HIV-1BAL for 3 hr. At the end of the incubation, cells were washed, re-plated and HIV replication was assessed by measuring p24 in the culture supernatant at day 0, 4 and 7 post exposure. HIV infection was also determined intracellularly by flow cytometry. Following 7 days of HIV exposure, mononuclear cells were permeabilized and stained with CD3-ECD, CD4-APC, CD8-PerCP-Cy5.5 and P24-RD-1 (KC57, Beckman Coulter) monoclonal antibodies (mAbs). Cell-culture supernatants were also assayed for P24 content at day 0, 4 and 7 in using an ELISA (Innogenetics).

**Statistical analyses.** P values were derived from either Chi-square analysis, for comparison of positive proportions, One-way ANOVA (Kruskal-Wallis test), followed by a Student’s t-test in the context of multiple comparisons, or a Spearman rank test for the correlations.
Results

Ad-specific but not poxvirus-specific CD4 or CD8 T cells reside in gut mucosal tissues

Cellular immunity (T cells) to different Ad serotypes is highly cross-reactive (31, 39). For these reasons, we have used the terminology of “Ad-specific T cells” and not “Ad5-induced T cells” to define T-cell responses specific to adenovirus. We have also used the terminology of “Smallpox-specific T cells” to define memory T-cell responses observed in subjects vaccinated with Smallpox and NYVAC-specific T-cell responses to define memory T-cell responses detected in subjects which received the DNA-C/NYVAC-C vaccine regimen.

To evaluate the pre-existing immunity against poxvirus and Ad vector-based vaccines, the anatomic distribution of Ad-specific versus Smallpox-specific CD4 and CD8 T cells, we analyzed the presence of Ad-specific and Smallpox-specific CD4 and CD8 T cells in mononuclear cell populations isolated from blood and gut mucosal tissues (ileum and rectum). To assess virus-specific T-cell responses, blood mononuclear cells isolated from both blood and gut mucosal tissues (ileum and rectum) from HIV-uninfected individuals were labeled with CFSE and stimulated for 6 days with Ad5 or empty NYVAC vectors. At the end of the stimulation period, cells were stained with CD3, CD4 and CD8 mAbs. As shown in representative flow cytometry profiles of subjects #025 and #014 (Fig. 1A and 2A) and in the cumulative data of the 25 subjects studied (Fig. 1B-C and 2B-C), Ad-specific CD4 and CD8 T cells were detected in both blood and gut mucosal tissues. The proportion of individuals having detectable Ad-specific CD4 and CD8 T cells (referred to as responders) were similar in the two compartments, i.e. 56% in blood versus 58.3% in the rectum and 62.5% in the ileum for CD4 T-cells and 48% in blood versus 48% in the rectum and 50% in the ileum for CD8 T-cells (Fig. 1B and 2B). The levels of T-cell proliferation (percentage of CFSE low cells among total CD4 or CD8 T cells) were similar among the two compartments for both Ad-specific CD4 and CD8 T cells (Fig. 1C and 2C). These
results demonstrated that pre-existing Ad-specific CD4 and CD8 T cells resided also in gut mucosal tissues. Interestingly, the frequencies of CD4 T-cell proliferating in response to Ad5 stimulation correlated between blood and gut tissues (rectum: $P=0.001$ and ileum: $P=0.01$) indicating that the evaluation of immunological measures of Ad-specific CD4 T-cell responses in blood might reflect those of Ad-specific CD4 T-cells resident in gut mucosal tissues.

As shown in one representative individual (Fig. 1A and 2D) and in the cumulative data (Fig. 1D-E and 2E-F) Smallpox-specific CD4 and CD8 T cells were mainly detected in blood and very rarely (1 out of 12) in gut mucosal tissues. The proportion of Smallpox-specific T cell responders was dramatically reduced (4% and 0%) for both CD4 T-cell responses in gut mucosal tissues (rectum and ileum, respectively) as compared to blood: (52%) ($P=0.0002$ and $P<0.0001$) and CD8 T-cells: (12% blood versus 0% in gut tissues; $P>0.05$) (Fig. 1D and 2D). Therefore, memory Smallpox-specific T-cell responses studied more than 30 years after vaccination were rarely detected in gut mucosal tissues.

Frequencies of Ad-specific CD4 T cells in blood and gut mucosal tissues do not correlate with Ad5-specific neutralizing antibody titers

Recently, two studies have found no correlation between Ad5-NAbs titers and levels of proliferating Ad-specific CD4 T cells isolated from blood before or after Ad5 immunization (16, 27). It was then relevant to determine whether Ad5-NAbs titers correlated with the presence and/or the proliferative capacity of Ad-specific CD4 T cells isolated from gut mucosal tissues in adenovirus-naturally infected volunteers. To address this issue, Ad5-NAbs titers were determined for each volunteer as previously described (30). The volunteers were stratified based on their Ad5 serostatus into two groups: 1) Ad5 NAbs low ($<$18) and 2) Ad5 NAbs high ($>$18) (6, 22). The proportion of Ad-specific responders (individuals with CD4 T-cell proliferation) was similar
between volunteers with lower and higher titers of Ad5 NAbs regardless of the anatomic compartment investigated (Fig. 3 A-C). In addition, the percentage of Ad-specific CD4 T-cells (percentage of CD3+CD4+ CFSE low cells) did not correlate with Ad5 NAbs titers in cells isolated from both blood and gut (Suppl. Figure 1).

**Anatomic Distribution of NYVAC- and HIV-specific CD4 and CD8 T cell populations**

Six individuals enrolled in a previous HIV vaccine trial, i.e. EV03 trial (19), who accepted colonoscopy and gut biopsies were investigated in both blood and gut for the presence of vector (NYVAC)-specific and insert-(HIV)-specific T-cell responses after vaccination. The individuals enrolled in the EV03 trial were immunized with 3 DNA-C injections (4 mg at week 0, 4 and 8) plus 1 NYVAC-C injection (10^7 pfu at week 24). The DNA and NYVAC vaccines expressed env, gag, pol and nef from the HIV-1 CN54 clade C isolate (10). The six individuals were studied about 2 years after receiving the last immunization. As for the Ad- and Smallpox-specific T-cells (Fig. 1 and 2), NYVAC- and HIV-specific T-cell responses were measured using antigen-specific proliferation in mononuclear cells isolated from both blood and gut mucosal tissues (ileum and rectum) of the same subjects. Cells were labeled with CFSE and stimulated for 6 days with empty (non expressing HIV antigens) NYVAC vector or with HIV peptide pools. At the end of the incubation period, cells were stained with CD3, CD4 and CD8 mAbs. As shown in a representative flow cytometry profile of subjects #1008U and #1037F (Fig. 4A and 5A) and in the cumulative data (Fig. 4B-C and 5B-C), NYVAC-specific CD4 and CD8 T cells were detected in both blood and gut mucosal tissues (ileum and rectum). The proportion of individuals having detectable proliferating NYVAC-specific CD4 and CD8 T-cells was similar in blood (83.3%) and gut tissues (rectum: 83.8%; ileum: 100%) (Fig. 4B and 5B). The magnitude of the T-cell
proliferation was similar between the two anatomic compartments for NYVAC-specific CD4 and CD8 T cells (Fig. 4C and 5C).

These results demonstrate that recent (about 2 years) intramuscular immunization with DNA-C plus NYVAC-C led to the generation of NYVAC-specific CD4 and CD8 T cells in blood and in gut tissues.

Along the same line, the generation of HIV-specific CD4 and CD8 T-cells were investigated in both blood and gut tissues. Interestingly, HIV-specific CD4 and CD8 T-cells were detected in both blood and gut mucosal tissues (ileum and rectum). Representative subjects (Subjects #1008U and 1037F) are shown in Fig. 4A and 5A. Cumulative data generated from the analysis of the 6 subjects are also shown (Fig. 4D-E and 5D-E). All subjects studied showed HIV-specific CD4 and CD8 T-cell responses in both blood and rectum, and 83.3% in the ileum (Fig. 4D and 5D), indicating that the DNA/NYVAC vaccine regimen induced the generation of HIV-specific CD4 and CD8 T-cell responses in blood and gut mucosal tissues. The frequencies of HIV-specific CD4 and CD8 T-cell responses tended to be higher in blood as compared to gut tissues (Fig. 4E and 5E). However, these differences were not significant (P>0.05). The percentage of HIV-specific CD4 T-cells was 17.6% in blood versus 9.7% in the rectum and 9.6% in the ileum, and the percentage of CD8 T-cells was 21.09% in blood, 10.8% in the rectum and 7.75% in the ileum (Fig. 4E and Fig. 5E).

**Proliferating Ad-specific and NYVAC-specific CD4 T cells express higher levels of α4β7 integrins than Smallpox-specific CD4 T cells.**

Tissue migration capacity of lymphocytes is orchestrated by the combined expression of specific integrins and chemokine receptors. The classical T-cell migration paradigm opposes
mucosal tropism to skin tropism (12, 17, 21, 24). In order to migrate to the small intestine, T lymphocytes have to express the chemokine receptor CCR9 (43) which recognizes CCL25, constitutively released in the small intestine (18, 42) but not in the colon (18, 29, 43), and to express α4β7 integrins, which binds MAdCAM-1 on endothelial cells (3, 41). In order to home to the skin, T lymphocytes have to express both CCR4 and cutaneous lymphocyte antigens (CLA) (7, 35). Of note, whereas CCR9 expression is required for small intestine migration, the chemokine receptor(s) involved in the migration to other regions of the gastrointestinal tract remains to be identified (26). We therefore examined the expression of CCR4, CCR9, α4β7 integrins and CLA in proliferating Ad-specific, Smallpox- and NYVAC-specific CD4 T cells isolated from blood of adenovirus naturally infected individuals (N=12), Smallpox (N=10) and NYVAC vaccinated (N=5) individuals. To perform this analysis, mononuclear cells were isolated from blood, labeled with CFSE and stimulated for 6 days with Ad5 or NYVAC vectors. The chemokine receptor and cell adhesion molecule profiles of proliferating T cells were determined by multiparametric flow cytometry. As shown in the representative individuals #ES0219 and #1029E (Fig. 6A and 6D) and in the cumulative data (Fig. 6E), proliferating (CFSE low cells) Ad-specific and NYVAC-specific CD4 T cells isolated from peripheral blood expressed higher levels of α4β7 integrins than Smallpox-specific CD4 T cells, i.e. Ad-specific 78.6%; versus Smallpox-specific 55.27% versus NYVAC-specific 84.62% (P=0.0002 and P<0.0001, respectively). In addition, Ad-specific, Smallpox-specific and NYVAC-specific CD4 T cells expressed intermediate levels of CLA, low levels of CCR9 and high levels of CCR4 (data not shown). These results suggested that proliferating Ad-specific, Smallpox-specific and NYVAC-specific CD4 T cells, isolated from blood have the potential to home to gut mucosal tissues. The reduced α4β7 integrins expression in smallpox-specific CD4 T cells (versus Ad-specific and
NYVAC-specific CD4 T cells) might potentially explain their distinct migratory capacity of these antigen-specific CD4 T cells. However, we cannot exclude that the differences between NYVAC-, Ad- and Smallpox-specific T-cells migratory capacity and specific immune responses might be attributed to increased time which had passed between antigen exposure and measurement of antigen-specific immune responses. Furthermore, the route of Smallpox vaccination (scarification) differed from those of Ad-natural infections (usually mucosal routes (36)) and NYVAC vaccination (intramuscular).

Ad-specific, Smallpox-specific and NYVAC-specific CD4 T cells isolated from peripheral blood and gut mucosal tissues express the HIV co-receptor CCR5

To determine whether proliferating vector-specific CD4 T-cells isolated from blood and mucosal tissues were susceptible to HIV infection, the levels of expression of the HIV co-receptor CCR5 were evaluated by multiparametric flow cytometry. Mononuclear cells isolated from blood and gut mucosal tissues (rectum and ileum) of Ad-naturally infected individuals (N=12), Smallpox vaccine recipients (N=10) and NYVAC vaccine recipients (N=5) were labeled with CFSE and stimulated for 6 days with Ad5 or NYVAC vectors. At the end of the stimulation period, cells were stained with CD3, CD4, CD8 and CCR5 mAbs. As shown in representative subjects #003 and #1029E (Fig. 7A and B) and in the cumulative data (Fig. 7C), proliferating Ad-specific, Smallpox-specific and NYVAC-specific CD4 T cells expressed high levels of CCR5 in both blood and gut compartments.

As expected the proliferating virus-specific CD4 T-cells from both blood and gut were able to support efficient HIV infection in vitro (Suppl. Figure 2).
Several replication-defective adenovirus and poxvirus vectors are among the most frequent strategies explored for the development of an HIV vaccine (23, 28, 38). There is large consensus within the scientific community on the importance of inducing vaccine responses both systemic (blood) and at mucosal sites, i.e. at the port of entry of HIV (4). In addition, the gut has also been shown to represent a major site for HIV replication and depletion of CD4 T-cells (5). For these reasons, it is important to characterize the vaccine-induced immune responses in different anatomic compartments including blood and mucosal tissues such as the gut.

The efficacy of a vaccine strategy can be substantially influenced by the presence of pre-existing immunity in the host (14). This issue is particularly important for vectors derived from adenovirus and poxvirus. There are 53 different Ad-serotypes which infect large proportion of the population depending on the geographic region and have tropism for different anatomic compartments (36, 40). While Ad-NAbs are serotype specific, Ads-specific T-cell responses are largely cross-reactive (15, 30, 31, 39). The issue of poxvirus pre-existing immunity concerns only certain age groups since Smallpox vaccination was stopped in the mid seventies.

In the present study, we have investigated a) the pre-existing Ad- and Smallpox-specific immunity in both blood and gut tissues, b) the poxvirus vector (NYVAC)- and HIV-specific T-cell responses in both blood and gut tissues following vaccination with a DNA-C/NYVAC-C regimen in healthy volunteers and c) the potential tropism of Ads-, Smallpox- and NYVAC-specific CD4 and CD8 T-cells.

Memory Ads-specific CD4 and CD8 T-cell responses were consistently found in both blood and gut tissues (rectum and ileum) in a large proportion of subjects studied while memory Smallpox T-cell responses were found in blood (predominantly CD4) and almost absent in gut tissues. The lack of detection of Smallpox-specific T-cells in the gut is either due to low
frequency of memory T-cells after at least 30 years from the time of Smallpox vaccination or to the route of immunization (see below).

Therefore, on the basis of these results Ad vector-based vaccines may induce cross-reactive T-cell responses both in blood and in gut tissues while Smallpox vaccines predominantly in blood. The data on Ad vectors are consistent with previous studies performed in blood in humans and in Non-Human Primates (NHPs) both in blood and gut tissues (2, 20).

The lack of detection of Smallpox-specific T-cells in gut tissues raised the question of whether poxvirus vectors induced T-cell responses, both vector-specific and HIV transgene-specific, were also induced at mucosal sites. Healthy volunteers immunized with the DNA-C/NYVAC-C vaccine regimen were simultaneously investigated in blood and gut (rectum and ileum) for the presence of vector- and HIV transgene-specific CD4 and CD8 T-cells. The results consistently indicated that this vaccine regimen was able to induce vector and HIV-specific CD4 and CD8 T-cell responses in both blood and gut tissues. The explanation of the differences between NYVAC and Smallpox vaccination in the induction of T-cell responses at mucosal sites remains unclear, but could be related to the degree of virus replication in human tissues as the Smallpox vaccine represents a replication competent virus while NYVAC is a non-replicating virus. In addition to what mentioned above, it is worth to mention that NYVAC was administered intramuscularly and NYVAC boost was preceded by three DNA administrations. In this regard, DNA priming substantially increases the immunogenicity of NYVAC resulting in a larger proportion of responders and in a T-cell response of several orders of magnitude greater than the administration of NYVAC alone (13).

Benlahrech et al. have recently shown that proliferating Ad-specific CD4 T cells isolated from blood of adenovirus-naturally infected volunteers expressed α4β7 integrins and CCR9 thus
indicating that Ad5-specific CD4 T cells may migrate to the small intestine (2). In this regard, Masek-Hammerman et al. have clearly shown in NHPs that Ad5-specific CD4 T-cells migrate to the small intestine and showed no differences in the mucosal tropism of Ad5-induced CD4 T-cells in monkeys in the presence or in the absence of pre-existing immunity to Ad5 (20). In the present study, we have shown that also poxvirus-specific T-cells induced by Smallpox and NYVAC vaccination expressed high levels of α4β7 integrins. However, blood Smallpox-specific CD4 T cells expressed significantly lower levels of α4β7 integrins than NYVAC-specific or Ad-specific CD4 T cells. The lower levels of expression of α4β7 integrins may partially explain the lack of detection of Smallpox-specific T-cells in the gut tissues. However, it is possible that the differences observed in the migratory capacity of NYVAC, Ad, and Smallpox-specific T-cells might result from the increased time which had passed between antigen exposure and measurement of antigen-specific immune responses. Both Ad- and poxvirus-specific CD4 T-cells expressed elevated and comparable levels of CCR5, and virus-specific CD4 T cells from both blood and gut tissues showed comparable susceptibility to HIV infection and support viral replication in vitro.

In conclusion, the present study clearly shows that immunization with the DNA/NYVAC vaccine regimen is capable of inducing HIV-specific T-cell immunity both systemically and at mucosal sites such as the gut which may serve as port of entry for HIV and as a major anatomic site for virus replication and depletion of CD4 T-cells (4), and in turn, the immune response triggered might play an important tolle in the control of HIV infection.
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Conflict of interest

The authors declare no conflicting financial interests.
References


Figure legends

Figure 1. Adenovirus-specific but not Smallpox-specific CD4 T cells are detected in gut-mucosal tissues. Mononuclear cells were isolated from blood and gut mucosal tissues (rectum and ileum), stained with CFSE and stimulated with Ad5 or NYVAC vectors. Following 6 days of incubation, cells were stained with CD3, CD4 and CD8 mAbs. (A) Flow cytometric profiles of proliferating Ad-specific and Smallpox-specific CD4 T cells from mononuclear cells isolated from blood and mucosal tissues (rectum and ileum). One representative subject (Subject #025) is shown. The flow cytometric profile of unstimulated cells (negative control) is also shown. Percentage of Ads-specific (B) and Smallpox-specific (C) CD4 T-cell responders (volunteers with CD4 T-cell responses). Frequencies of proliferating Ads-specific (D) and Smallpox-specific (E) CD4 T cells. Direct correlation between Ads-specific CD4 T-cell proliferation detected in blood and the rectum (F) or the ileum (G). Statistical analyses were performed using Chi-square methods for comparison of positive proportions, or using Student’s t-test for multiple comparisons or using Spearman rank test for the correlations.

Figure 2. Adenovirus-specific but not Smallpox-specific CD8 T cells are detected in gut mucosal tissues. Mononuclear cells were isolated from blood and gut mucosal tissues (rectum and ileum), stained with CFSE and stimulated with Ad5 or NYVAC vectors. Following 6 days of incubation, cells were stained with CD3, CD4 and CD8 mAbs. Flow cytometric profiles of proliferating Ads-specific (A) and Smallpox-specific (D) CD8 T cells from mononuclear cells isolated from blood and mucosal tissues (rectum and ileum). Two representative subjects (Subject #014 and #006) are shown. The flow cytometric profiles of unstimulated cells (negative control) are also shown. Percentage of Ads-specific (B) and Smallpox-specific (C) CD8 T-cell
responders (volunteers with CD8 T-cell responses). Frequencies of proliferating Ads-specific (E) and Smallpox-specific (F) CD8 T cells. Statistical analyses were performed using Chi-square methods for comparison of positive proportions, and Student's t-test for multiple comparisons.

Figure 3. Frequencies of Ad-specific CD4 T cells in blood and in mucosal tissues do not correlate with Ad5-specific neutralizing antibody titers. Ad5-NAbs titers were determined for each volunteer as described in material and methods. The volunteers were arbitrarily stratified by their Ad5 serostatus into two groups: Ad5-NAbs low (Ad5-NAbs <18; N=12) and Ad5-NAbs high (Ad5-NAbs >18; N=13). The percentage of Ad-specific CD4 T-cell responders (volunteers with Ad-specific CD4 T-cell proliferations) was compared in volunteers with low and high Ad5-NAbs titers for each compartment: peripheral blood (A), rectum (B) and ileum (C). Statistical analyses were performed using Chi-square methods for comparison of positive proportions.

Figure 4. NYVAC-specific and HIV-specific CD4 T cells home to gut-mucosal tissues. Mononuclear cells were isolated from blood and gut mucosal tissues (rectum and ileum) of subjects (N=6) vaccinated with DNA-C/NYVAC-C regimen in the EV03 study, stained with CFSE and stimulated with NYVAC empty vectors or HIV peptide pools encompassing env, gag, pol and nef regions. Following 6 days of incubation, cells were stained with CD3, CD4 and CD8 mAbs. (A) Flow cytometric profiles of proliferating NYVAC-specific and HIV-specific CD4 T cells in mononuclear cells isolated from blood and mucosal tissues (rectum and ileum). One representative subject (Subject #1008U) is shown. The flow cytometric profiles of unstimulated cells (negative control) are also shown. Percentage of NYVAC-specific (B) or HIV-specific (C) responders (volunteers with CD4 T-cell responses). Frequencies of proliferating NYVAC-specific (D) or HIV-specific (E) CD4 T cells. Statistical analyses were performed using Chi-
square methods for comparison of positive proportions, or by Student’s t-test in the case of multiple comparisons.

**Figure 5. NYVAC-specific and HIV-specific CD8 T cells home to gut mucosal tissues.**
Mononuclear cells were isolated from blood and gut mucosal tissues (rectum and ileum) of subjects (N=6) vaccinated with DNA-C/NYVAC-C regimen in the EV03 study, stained with CFSE and stimulated with NYVAC empty vectors or HIV peptide pools encompassing gag, pol, nef and env regions. Following 6 days of incubation, cells were stained with CD3, CD4 and CD8 mAbs. (A) Flow cytometric profiles of proliferating NYVAC-specific and HIV-specific CD8 T-cells in mononuclear cells isolated from blood and mucosal tissues (rectum and ileum). One representative subject (Subject #1037F) is shown. The flow cytometric profiles of unstimulated cells (negative control) are also shown. Percentage of NYVAC-specific (B) or HIV-specific (C) responders (volunteers with CD8 T-cell responses). Frequencies of proliferating NYVAC-specific (D) or HIV-specific (E) CD8 T cells. Statistical analyses were performed using Chi-square methods for comparison of positive proportions, or by Student’s t-test in the case of multiple comparisons.

**Figure 6. Adenovirus-specific and NYVAC-specific CD4 T cells express higher levels of α4β7 integrins than Smallpox-specific CD4 T cells.** Mononuclear cells were isolated from blood, stained with CFSE and stimulated with Ad5 or NYVAC vectors. Following 6 days of incubation, cells were stained with CD3, CD4, CD8, α4/β7 integrins mAbs. Flow cytometric profiles of proliferating Ads-specific (A and B), Smallpox-specific (A and B) and NYVAC-specific (C and D) CD4 T cells (CFSE low cells) expressing β7 integrin or co-expressing α4 and
β7 integrins are shown. Black dots correspond to CD3+CD4+ CFSE high cells and red dots to CD3+CD4+ CFSE low cells. Cumulative data of proliferating Ads-specific, Smallpox-specific or NYVAC-specific CD4 T cells (CD4 CFSE low cells) expressing α4β7 integrins (E).

**Figure 7.** Proliferating Ad-specific, Smallpox-specific, and NYVAC-specific CD4 T cells express high levels of HIV co-receptor CCR5. Mononuclear cells were isolated from peripheral blood and gut mucosal tissues (Rectum and Ileum), stained with CFSE and stimulated with Ad5 or NYVAC vectors. Following 6 days of incubation, cells were stained with CD3, CD4, CD8 and CCR5 mAbs. Flow cytometric profiles of proliferating (CFSE low cells) Ads-specific (A), Smallpox-specific (A), and NYVAC-specific (B) CD4 T-cells in mononuclear cells isolated from blood and mucosal tissues (Rectum and Ileum) expressing CCR5. Two representative subjects (Subject #003 and #1029E) are shown. The flow cytometric profiles of unstimulated cells (negative control) are also shown. Percentage of Ads-specific, Smallpox-specific, and NYVAC-specific CD4 T-cells (CD3+CD4+ CFSE low cells) expressing CCR5 (C). P values were determined using Student’s t-test.