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HIV-1 Vaccination Administered Intramuscularly Can Induce Both Systemic and Mucosal T Cell Immunity in HIV-1-Uninfected Individuals

Luwy Musey,* Yan Ding,† Marnie Elizaga,*† Richard Ha,‡ Connie Celum,* and M. Juliana McElrath2*†

A vaccine regimen that can rapidly control HIV-1 replication at the site of exposure following sexual contact is likely to be the most effective in preventing HIV-1 infection. As part of a larger, phase II clinical trial, we evaluated the ability of a recombinant canarypox HIV-1 vaccine to induce CTL that can be detected in both the systemic and mucosal compartments following i.m. immunization in 12 low- and high-risk HIV-1 seronegative volunteers. In the 7 volunteers receiving four immunizations with live recombinant canarypox ALVAC-HIV vaccine with or without rgp120/SF-2, HIV-1-specific CTL were detected in the blood of 5 (71%) and in the rectum of 4 (57%). CTL responses were observed in both risk strata. In contrast, 5 volunteers receiving placebo had undetectable responses in both compartments. Vaccine-induced, HIV-1-specific effector activities included IFN-γ secretion and class I MHC-restricted CD8+ CTL. Rectal and systemic CD8+ CTL clones established in 1 vaccine recipient revealed similar Env-specific responses and MHC restriction. These findings indicate that parenteral vaccination can induce HIV-1-specific CTL that localize to sites of HIV-1 acquisition, where their presence may be critical in the control of initial viral replication and eventual dissemination. Determination of the optimal strategy to induce mucosal T cells requires future clinical studies. The Journal of Immunology, 2003, 1710: 1094–1101.

The global HIV-1 epidemic continues unabated (1). The best long-term strategy to control the spread of infection is through the development and use of a safe and effective HIV-1 vaccine. Components of immunity presumed, but not proven, to correlate with vaccine-induced protection include broadly reactive neutralizing Abs to HIV-1 isolates commonly transmitted by sexual transmission, and T cells, both helper and cytotoxic, that recognize epitopes in association with MHC molecules. Numerous studies attest to the critical role of class I MHC-restricted CD8+ CTL in controlling HIV-1 infection in humans and SIV or simian/human immunodeficiency virus infection in macaques (2–6). HIV-1-specific CD8+ CTL have also been demonstrated in individuals repeatedly exposed to HIV-1 for years who appear to resist infection, thus suggesting a contributing role of CTL in defense against HIV-1 (7–9).

Because HIV-1 infection is acquired primarily through mucosal routes, the presence of virus-specific CD8+ CTL at the site of viral entry may be critical in restricting viral dissemination to the lymphoid tissue and the blood. Previous studies have identified SIV- and HIV-specific CTL in the genital tract of SIV-infected macaques (10, 11), HIV-infected humans (12, 13), and in HIV-1-exposed seronegatives (14). Recent vaccine studies have implicitly demonstrated the protective role of CD8+ CTL in macaques first immunized with SIV-, simian/human immunodeficiency virus-, or HIV-2-based vaccines, and subsequently challenged mucosally with more virulent SIV strains (15–19). Moreover, studies in mice vaccinated with recombinant vaccinia encoding HIV-1 env demonstrate that CTL, especially those located in mucosal tissues, can mediate protection against mucosal challenge (20). We have demonstrated that HIV-1 infection induces class I MHC-restricted CD8+ CTL in the genital tract and rectal mucosa that are capable of recognizing HIV-1-infected cells and secreting antiviral cytokines such as IFN-γ (12, 21). These results suggest that mucosal CTL may contain viral replication and thus, if elicited by vaccination, may provide immune surveillance upon exposure to HIV-1 by sexual contact.

In this study, we explored the possibility that HIV-1 vaccination can elicit virus-specific CTL at mucosal sites. Canarypox vectors containing HIV-1 gene inserts have undergone extensive testing in phase I–II trials. Notably, live recombinant canarypox ALVAC-HIV vaccine (vCP205),3 containing HIV-1 genes encoding env, gag, and portions of pol, has induced cumulatively HIV-specific CD8+ CTL in the peripheral blood of 30–50% of HIV-seronegative vaccine recipients (22–24). However, vaccine-induced mucosal CTL responses have not been assessed in these studies, and heretofore it has been unclear whether examination of sufficient numbers of mucosal T cells was feasible without subjecting volunteers to more invasive procedures to obtain the relevant mucosal samples. Thus, within a phase II clinical trial evaluating the safety and immunogenicity of vCP205 in low- and high-risk HIV-seronegative volunteers (24), we embedded a smaller investigation to examine the induction of both systemic (peripheral blood) and mucosal (cervical, rectal) CTL in consenting volunteers from our clinic. Remarkably, we found that live vector immunization can

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3 Abbreviations used in this paper: vCP205, live recombinant canarypox ALVAC-HIV vaccine; B-LCL, B lymphoblastoid cell line; rVV, recombinant vaccinia virus; M01, multiplicity of infection; SFC, spot-forming cell.

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induce CTL that not only circulate in peripheral blood but also localize to the mucosa. This study establishes a framework now to explore the optimal approach to elicit and measure the kinetics of these responses.

Materials and Methods

Study population

Sixty-three subjects were enrolled by the University of Washington AIDS Vaccine Evaluation Unit and the Seattle HIVNET as part of a multicenter, double-blind randomized phase II HIV-1 vaccine trial (AEGV 202/HIVNET 014) conducted jointly by the two networks. Healthy, HIV-1-uninfected adult volunteers reporting activities consistent with lower or higher risk of acquiring HIV-1 infection were eligible to participate in the study, and detailed enrollment criteria have been published (24). Vaccine recipients were injected i.m. with vCP205 with or without HIV-1 gag rp120 at 0, 1, 3, and 6 mo. Placebo recipients were administered either vehicle or saline at some time points. The ALVAC-HIV-vCP205 (Aventis Pasteur, Paris, France) is a recombinant canarypox vector vaccine containing the following HIV-1 genes: HIV-1 gag envelope gp120, HIV-1 L-A1-L anchoring transmembrane gp41, and the HIV-1 L-A1-L genes encoding the entire Gag protein and a portion of pol encoding the HIV-1 protease. Each 0.5-ml dose contained 5 × 10^5 tissue culture-infective dose of virus. The rp120 vaccinia (Chiron, Emeryville, CA) was given as 0.5-ml doses containing 50 μg of Ag in combination with MF59 adjuvant emulsion (24).

We invited the first 36 Seattle volunteers enrolled in the clinical trial to participate in an ancillary study to evaluate both mucosal and systemic T cell immunity following vaccination. Autologous EBV B lymphoblastoid cells (B-LCL) were derived from consented volunteers. Vaccine-induced CTL were measured in peripheral blood at days 98 and 182 of the study (2 wk after the second and third immunization). We then selected 24 volunteers with viable B-LCL, 12 with and 12 without detectable CTL in the blood, to participate further in the mucosal studies. Of note, to maintain the vaccine-induced, the CTL findings in peripheral blood were not revealed to the volunteers, investigators, and clinicians directly involved in the ancillary study. Also, the laboratory technical personnel involved in the mucosal CTL study were different and blinded to the peripheral blood CTL findings. Of the 24 selected, 14 consenting volunteers provided blood and/or either rectal (male subjects) or cervical (female subjects) specimens at 9, 12, and 15 mo postenrollment, corresponding to 3, 6, and 9 mo after the last immunization, respectively.

All study participants received HIV-1 risk reduction counseling and HIV-1 testing at 3-mo intervals. A testing algorithm was used to discriminate between vaccine-induced Ab responses and acquisition of infection, which consisted of serological followed by licensed HIV-1 RNA testing by RT-PCR assays. The University of Washington Human Subjects Review Committees approved all aspects of the study.

Isolation of blood and mucosal T lymphocytes

PBMC were isolated by Ficoll-Hypaque density centrifugation. Cervical T cells were obtained following one 90° turn of a cytobrush and isolated as previously described (12). Samples with visible or microscopic blood contamination were discarded. Rectal biopsies were obtained immediately in fresh transport medium (RPMI 1640 supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (2 μg/ml)) and processed within 2 h after collection. The tissue was washed five times with cold PBS, minced, and then washed again three times with cold RPMI 1640 with 10% human AB serum, penicillin (100 U/ml), streptomycin (100 μg/ml), fungizone (2 μg/ml), and 2 mM glucose. They were then placed in 2 ml of fresh medium incubated for 24 h at 37°C and 5% CO_2 to allow T cells to migrate from the tissue into the culture medium. The cell mixture was then treated twice with collagenase D (Boehringer Mannheim, Indianapolis, IN) (final concentration, 2 mg/ml) for 10 min at 37°C and 5% CO_2 to further disperse the rectal T cells from the tissue into the medium. The rectal T cells were then passed through a 140-μm screen to separate them from tissue debris and epithelial cells. Flow cytometric analysis of mononuclear cell phenotypes was performed, as previously described (25).

Generation and testing of CTL lines and clones

Cell lines from cervical and rectal specimens were established in 96-well plates, with initial cultures containing ~100-10,000 mononuclear cells/well. Two million PBMC were cultured per well in a 24-well plate. T cells from these specimens were then stimulated with autologous irradiated monocytes previously infected with a recombinant vaccinia virus (rVV) vector expressing HIV-1 env<sub>gp160</sub>/gag<sub>pol</sub>/pro<sub>tease<sub>pol</sub></sub> (vP1291) (kindly provided by Dr. J. Tartaglia (Virogenetics, Troy, NY) at a multiplicity of infection (MOI) of 5, as previously described (12), autologous irradiated PBMC feeder cells, and human rIL-2 (final concentration of 50 U/ml). After 7–10 days of culture, wells demonstrating cell growth were expanded into 12-well tissue culture plates by adding anti-CD3/4b (Santa Cruz Biotechnology, Santa Cruz, CA) at a concentration of 0.5 μg/ml (kindly provided by Dr. W. J. Wong (Massachusetts General Hospital, Boston, MA)) and irradiated allogeneic PBMC feeders (2 × 10^5 /well). After 10–14 days, cultures were tested for the detection of HIV-1-specific CTL in a standard chromium release assay at various E:T ratios.

Target cells were autologous or allogeneic B-LCL infected with IVV expressing either HIV-1<sub>env</sub>envelope (vP1174), HIV-1<sub>gag</sub> gag (vP1287), HIV-1<sub>pol</sub> pol (vP1288), or the lacZ gene control (v-Lac) (all provided by Dr. J. Tartaglia (Virogenetics)) at a MOI of 1 (12). Cr release was measured using a TopCount (Packard, Meriden, CT). In all CTL experiments, spontaneous Cr release in target cells cultured with medium alone was <25% of maximum lysis of target cells cultured with 5% Triton X-100 (Sigma-Aldrich, St. Louis, MO). The percent lysis was calculated as follows: 100 × (test release – spontaneous release)/maximum release – spontaneous release. A response was considered positive if the percent specific lysis (lysos observed against HIV-1 gene product minus lysis observed against the vaccinia or peptide control) was ≥10% in at least two E:T ratios.

CTL clones were established from HIV-specific T cell lines, as previously described (21). Two time points were used for the CTL line or clone, specific lysis of B-LCL targets from donors with a partial class I allele mismatch was compared with lysis of autologous target cells. Class I HLA serological typing of donor PBMC was performed by the Puget Sound Blood Center (Seattle, WA). To determine the T cell subset mediating cytolysis, CD4<sup>+</sup> or CD8<sup>+</sup> T cells were positively selected using CD4<sub>microbeads</sub> or CD8<sub>microbeads</sub> (Miltenyi Biotec, Auburn, CA), respectively. Fine epitope mapping was performed with small overlapping peptides varying between 8 and 20 aa in length spanning HIV-1 env (kindly provided by the National Institutes of Health AIDS Research and Reference Reagent Program (Bethesda, MD)) or synthesized at the Shared Resources Facility at Fred Hutchinson Cancer Research Center.

IFN-γ ELISPOT assay

IFN-γ spot-forming cells (SFC) were measured using modifications of a previously described method (26). Primary (clone 1-D1K; Mabtech, Nacka, Sweden) and secondary IFN-γ mAbs (biotinylated clone 7-B6;1, Mabtech), and the conjugate (avidin peroxidase complex; Vectastain ABC Elite kit; Vector Laboratories, Burlingame, CA) and substrate (Vectastain AEC substrate) were used according to the manufacturer’s instructions. Autologous B-LCL stimulated with various rVV expressing HIV-1 env, gag, pol, or the irrelevant lacZ gene were used as stimulators at a concentration of 20,000 per well. Responding cells were T cells from either freshly isolated or 14-day-stimulated T cell lines from blood, cervical, or rectal tissues, plated in triplicate at 50,000 cells/well. Negative controls included responding cells without and with stimulator cells infected with IVV expressing lacZ. Responding cells stimulated with either medium alone (control, 2 μg/ml; Sigma-Aldrich) served as the positive controls. An assay was considered positive if the number of SFC among cells stimulated with HIV-1<sub>env</sub> protein was ≥10 per 10^5 mononuclear cells and two times greater than the number of spots measured in cells cultured with the vaccinia control. This criteria was established based upon testing of PBMC and mucosal cells (semen and cervical)from 10 uninfected non-vaccinated low-risk volunteers in the same assay.

Results

Among the 14 volunteers who had viable B-LCL and who provided both blood and mucosal specimens, 12 were male and 2 were female. At study entry, 7 of the males reported high-risk sexual practices, primarily receptive anal sex. Two male subjects were excluded from the immunological evaluation due to contamination of rectal T cell cultures. The baseline characteristics of the 12 remaining individuals with evaluable blood and mucosal specimens, including their HIV-1 risk activities and immunization treatment code (as revealed at the end of the study) are shown in Table I. Seven volunteers received vaccine (vCP205 alone in 5; vCP205 and rp120/SF-2 in 2), and 5 received placebo. All 12 study participants were HIV-1 seronegative at enrollment and remained HIV-1 uninfected throughout the study period.

Acquisition of the mucosal specimens was well tolerated among all subjects, and no adverse reactions occurred. Cervical and rectal
samples yielded between 0.1 and 5 million mononuclear cells per total specimen. When sufficient cell numbers allowed limited surface phenotypic assessment by flow cytometry, the analysis indicated that ~60% of the total cell population were CD3+ T cells, and the majority (75–85%) of these expressed TCRαβ (data not shown).

**Induction of CTL responses after four immunizations**

The first evaluation of vaccine-induced immune responses in both the blood and mucosal tissues was performed at day 270, corresponding to 3 mo after the fourth and last immunization. Cytolytic activities are shown in 3 representative donors in Fig. 1, and findings in the 12 subjects are summarized in Table I. Five of the 7 (71.4%) vaccine recipients exhibited peripheral blood CTL responses recognizing either HIV-1 Gag only (n = 2), or both HIV-1 Env and Gag (n = 3) (Table I; Fig. 1). Lysis by blood effectors of targets expressing HIV-1 epitopes ranged from 19 to 39% (13–24% specific lysis, subtracting from the control target cell lysis) at an E:T ratio of 20:1. In contrast, no HIV-1-specific CTL were detected from rectal mucosal tissues in 3 of 6 (50%) vaccine recipients with evaluable mucosal samples. One subject (202FJ1) also demonstrated a Gag-specific response (Table I; Fig. 1). Cervical T cells from the 1 female vaccine recipient did not demonstrate CTL. The levels of specific lysis noted in the rectal mononuclear cells were similar to those in peripheral blood, with specific lysis of the HIV-1-expressing targets at an E:T ratio of 20:1, ranging from 11 to 32%. None of the 5 placebo recipients had detectable HIV-1-specific mucosal CTL (Table I). Thus, these results indicate that immunization with vCP205 can induce HIV-1-specific CTL that can be detected in the rectal mucosa, and that this can be accomplished by parenteral injection.

Of note, HIV-1-specific CTL were detected at day 270 in one or both compartments in all 7 volunteers: both compartments in 1, blood only in 4, and rectal tissue only in 2. In addition, whereas both Env- and Gag-specific responses were observed in both compartments, Gag-specific T cells occurred more commonly in blood than in the rectal tissue (Table I; Fig. 1). Taken together, these findings at one study time point suggest that examination of CTL in the blood alone may underestimate the frequency of responders to this vaccine regimen.

**Persistence of vaccine-induced CTL responses**

We next sought to determine the durability of the T cell responses over the subsequent 6–9 mo following the last (fourth) immunization. These studies, performed before unblinding the treatment code, were focused at day 340 on 5 consenting volunteers of the 7 who previously demonstrated a positive CTL response at day 270, and at day 455 on 4 consenting volunteers (see Table I for specific subjects tested). Two of the previous 5 responders at day 270 had detectable HIV-1-specific CTL responses at day 340. T cells derived from blood and the rectum recognized determinants within HIV-1 Env in these 2 subjects, 202FJ1 (not shown) and 202FJ9 (Fig. 2). Subject 202FJ9 had both Gag- and Env-specific CTL in the blood sample analyzed at 3 mo after the last immunization (day 270) (Fig. 2). However, this volunteer maintained detectable Env-

### Table I. Detection of HIV-1-specific CTL among study participants

<table>
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<tr>
<th>Treatment/Volunteer</th>
<th>HIV-1 Risk</th>
<th>Specimen</th>
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<th>Env</th>
<th>Gag</th>
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**ALVAC vCP205 + placebo**

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<th>LacZ</th>
<th>Env</th>
<th>Gag</th>
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**ALVAC vCP205 + SF2 rgp120 in MF59**

<table>
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<th>Specimen</th>
<th>LacZ</th>
<th>Env</th>
<th>Gag</th>
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a Data shown are percent cytolysis measured at an E:T ratio of 20:1 in a 5-h chromium release assay using autologous B-LCL targets infected with rVV expressing the control LacZ protein (v-Lac), HIV-1 Env (v-Env), and HIV-1 Gag (v-Gag). For volunteer 202FJ1, lysis by PBMC is shown at 5:1.

b Positive responses are designated in bold and defined as percent lysis ≥10% after subtracting lysis of the control targets, at two E:T ratios.

 Also analyzed at day 340.

d Also analyzed at day 455.

e The rectal specimen was not evaluated on the first visit because of culture contamination. A repeat specimen was obtained later, and CTL were detected.
Additional analyses of virus-specific immune responses in 4 volunteers (denoted in Table I) were performed at day 455, corresponding to 9 mo after the last immunization. No HIV-1-specific T cells were detected from blood or rectal tissues from any of the 4 subjects, which included both 202FI1 and 202FJ9 who had demonstrated responses at the previous two time points (data not shown). These results indicate that both systemic and mucosal responses induced by the vCP205 immunogen uncommonly persist at detectable levels, which is consistent with findings in the majority of vaccine recipients who manifested CTL in peripheral blood once but not multiple time points in a previous trial with vCP205 (23).

**Functional characteristics of the vaccine-induced mucosal CTL**

To further characterize the HIV-1-specific CTL in the two compartments, additional analyses were performed in 3 volunteers in whom sufficient specimens were available at either day 270 or 340. To determine the T cell subset mediating the HIV-specific CTL, CD8\(^+\) and CD4\(^+\) cells were positively selected from the cell lines derived from the rectal tissue and blood, and the effector populations were tested separately for lysis in the chromium release assay. Cytolytic activities were primarily mediated by CD8\(^+\) T cells, as illustrated in Fig. 3A in rectal CD8\(^+\) T cells from subject 202FJ9 recognizing HIV-1 Env-expressing target cells. Similar patterns were observed in peripheral blood (data not shown).

Mucosal T cells from the same line were also tested for their ability to secrete IFN-\(\gamma\) in response to HIV-1 Env-expressing stimulator cells using an ELISPOT assay. Of note, insufficient cell numbers precluded testing freshly isolated mucosal mononuclear cells in this assay, although a low level of Env-specific IFN-\(\gamma\)-secreting cells (30 SFC/10\(^5\) PBMC) was measured from freshly isolated blood. As shown in Fig. 3B, CD8\(^+\) T cells and, to a lesser extent, CD4\(^+\) T cells secreted IFN-\(\gamma\) when stimulated with autologous B-LCL infected with rVV expressing HIV-1 Env. In contrast, IFN-\(\gamma\)-SFC were rare (<10 SFC/10\(^5\) rectal mononuclear cells) in the rectal T cells following stimulation with B-LCL infected with the control rVV (Fig. 3B) and in the unstimulated PBMC and PBMC stimulated with the lacZ-expressing vaccinia control. Interestingly, in 1 subject (202FI1), mucosal effector T cells lysed autologous target cells expressing HIV-1 env (Fig. 1) but failed to secrete IFN-\(\gamma\) SFC after stimulation with APC expressing the same HIV-1 gene product (not shown). These results

**FIGURE 1.** Induction of systemic and mucosal CTL in three vaccine recipients, 202JQW, 202F6, and 202F11. The bars indicate percent lysis by Ag-stimulated T cell lines of autologous B-LCL expressing the control LacZ (v-Lac), HIV-1 Env (v-Env), and HIV-1 Gag (v-Gag) proteins at the designated E:T ratio.

**FIGURE 2.** HIV-1-specific CTL in subject 202FJ9 from blood and rectum at 3 mo (day 270) and 6 mo (day 340) after four immunizations with vCP205. PBMC from day 270 (left) and day 340 (middle), as well as rectal T cells from day 340 (right) were stimulated in vitro (see Materials and Methods) and tested for lysis of autologous B-LCL infected with rVV expressing LacZ (v-Lac), HIV-1\(_{\text{LA1}}\) Gag (v-Gag), and HIV-1\(_{\text{MN}}\) Env (v-Env) at different E:T ratios.
indicate that CD8+ T cells mediate the predominant vaccine-induced T cell responses, which are capable of lysing targets expressing HIV-1 epitopes and, in some instances, secreting IFN-γ.

**MHC restriction analysis and epitope mapping**

To determine whether the vaccine-induced CD8+ T cell responses were restricted by class I molecules, we tested the capacity of the HIV-1-specific CTL cell lines from subject 202FJ9 to recognize and lyse Env-expressing autologous B-LCL and/or partially mismatched B-LCL at the HLA-A and -B alleles. As shown in Fig. 4, both PBMC and rectal T cells lysed autologous Env-expressing targets as well as allogeneic targets from 2 donors (Allo#1 and Allo#3) sharing the class I HLA-A24 allotype. The MHC restriction pattern was later confirmed using target cells pulsed with the minimal envelope epitope recognized by PBMC and rectal T cells. Thus, HIV-1-specific responses induced in both systemic and rectal compartments were MHC-restricted and, in this case, to the same HLA class I molecule.

To ascertain which the Env epitope(s) recognized by the mucosal CTL were similar to that recognized by the blood CTL, the Env-specific cell lines from subject 202FJ9 were expanded into T cell clones (12, 21). Twenty-seven clones were successfully generated, 18 from PBMC and 9 from rectal T cells. To define the minimal epitope recognized, as shown for a representative blood clone in Fig. 5, autologous targets were pulsed with 8- to 20-mer peptides spanning the HIV-1MN Env protein (of note, some gaps occurred, because not all peptides were available from the repository). The CTL clones recognized one peptide pool within the first 140 a.a. of gp120 (Fig. 5A). When the clones were tested for lysis of targets pulsed with the individual peptides from this pool, a single response was detected to targets expressing gp120 a.a. 101–111, whose sequence is QMHEDIISLW. We repeated the MHC restriction analysis in 2 blood CTL clones and 1 mucosal CTL clone, and confirmed that the responses to the 10-mer (QW10) was restricted by HLA-A24 (data not shown). Individual clones also recognized the MW9, QL9, and HQ9 peptides. Thus, systemic immunization with vCP205 can induce CD8+ CTL detectable in both blood and mucosa.

**FIGURE 3.** HIV-1-specific CTL derived from rectal T cells of subject 202FJ9 are mediated by CD8+ T cells and secrete IFN-γ following Ag stimulation. A, Undepleted, CD8+ and CD4+ rectal T cells were tested for lysis of autologous B-LCL expressing HIV-1 Env (v-Env) or the control protein, LacZ (v-Lac), and results are shown at an E:T ratio of 5:1. B, Rectal T cells (undepleted, CD8+, and CD4+) were examined for IFN-γ secretion in an ELISPOT assay following stimulation with either v-Lac- or v-Env-infected autologous B-LCL. The error bars reflect the SEM of triplicate measurements.

**FIGURE 4.** Envelope-specific CD8+ CTL derived from the rectum and blood of subject 202FJ9 are restricted by the same class I molecule, HLA-A24. Rectal (A) and blood (B) Env-specific T cell lines were tested for lysis of autologous and partially class I HLA-mismatched allogeneic B-LCL at an E:T ratio of 5:1. The y-axis indicates the HLA class I serological type for the autologous targets and the shared class I allele in each allogeneic donor (Allo#1, Allo#2, Allo#3, and Allo#4).
that can be defined at the clonal level with similar epitope specificities and class I MHC restriction.

**Discussion**

We demonstrate in this study for the first time that parenteral HIV-1 immunization can induce HIV-1-specific CTL that can be detected in the rectal mucosa of HIV-1-uninfected vaccine recipients. The responses observed following i.m. injection of recombinant canarypox vector containing HIV-1 gene inserts were cytolytic for targets expressing HIV-1 Gag, Env, or both, and in some cases capable of secreting IFN-γ upon Ag stimulation. The ability to further detect vaccine-induced CTL with similar specificity and MHC restriction at the clonal level from both blood and the rectal mucosa provides confirmatory evidence that the mucosal responses were induced systemically.

We emphasize that this was a feasibility study designed to understand whether mucosal responses could be detected following HIV-1 vaccination. Initial concerns that blood cells may contaminate mucosal biopsies were alleviated by overall scant bleeding during the procedure and repeated washing of the rectal tissue before dispersion. Also, if the CTL activity in the mucosa was due to blood contamination, then a similar pattern of reactivity would be expected in the paired blood sample, but this was not observed. However, there were several limitations of the study that precluded more detailed examination and accurate quantitation of the CTL responses, as can be more easily accomplished in murine and macaque models and in HIV-1-infected patients. First, this investigation was nested into a larger phase II trial, which enrolled volunteers of diverse HLA type and which imposed constraints on the blood volume available and the frequency of mucosal sampling for our analyses. The mononuclear cell numbers isolated from the mucosal specimens were logarithmically fewer than from peripheral blood. As a result, we were unable to incorporate ex vivo flow cytometric evaluations such as tetramer binding and intracellular cytokine staining to quantify, phenotype, and compare responses within the mucosal and systemic compartments. Because of the labor intensity of the mucosal studies, we optimized the possibility of detecting a mucosal response by focusing the effort in large part.
on persons with known previous systemic responses, particularly due to selection of peripheral responders for the mucosal study. In addition, the mucosal CTL responses were primarily Env-specific (3 of 3 responders) rather than Gag-specific (1 of 3 responders). There is some chance that the Env-specific responses that we were not able to characterize at the clonal level may represent Ab-dependent cellular cytotoxicity or NK activity. However, this possibility is unlikely, because the reaction mixtures were not supplemented with gp120-specific Abs, and NK cells were not commonly expanded in the mucosal cell lines. Taken together, our study now provides a framework to proceed further using more precise analyses to quantify and phenotype mucosal CTL and to define the kinetics of these responses prospectively. Plans are underway to do so with HIV-1 candidate immunogens recently reported to induce strong and hopefully more durable CD8+ T cell responses in immunocompetent adults.

The mechanisms implicated in T cell homing to the mucosa following parenteral immunization deserve further investigation. We previously demonstrated identical clonotypes of HIV-1-specific CTL from blood and the rectal and genital mucosa (21) in HIV-1-infected patients who acquired infection by unprotected sexual activities. In this case, the CD8+ CTL can circulate between the systemic to mucosal compartments, and this may indicate trafficking back to the mucosal site where Ag is first encountered. Moreover, because the intestinal tract is a major HIV-1 reservoir (27), it is not surprising that memory CTL would localize there. There is the possibility that high-risk activities (especially receptive anal intercourse) may have primed vaccine recipients for HIV-1-specific T cell responses at the mucosal sites. However, previous mucosal exposure was not a prerequisite for identifying mucosal CTL in some of the vaccine recipients reported in this study. Of note, recent vaccine studies in nonhuman primates provide evidence to support our findings, with SIV-specific cells identified in the intestinal and vaginal mucosa (28, 29). Thus, our results suggest that vaccine-induced immunity may not be restricted within the compartment where it was first induced.

The presence of HIV-specific, activated effector T cells at mucosal surfaces may be critical in rapid control of viral infection after exposure. The success of vaccine candidates may best correlate with their ability to induce such responses mucosally, as has been reported in recent primate studies demonstrating that only animals with local HIV- or SIV-specific IgG, IgA, or CTLs were protected against challenge with a more virulent strain (19, 30–35). It is conceivable that, although i.m. immunization can induce CTL that can be detected in the mucosal compartment, this may not be the optimal route to do so, and more targeted routes of mucosal immunization may be more successful. This appears to be the case in nonhuman primate studies (36, 37). In addition, evaluation of new vectors and adjuvants that can be delivered by multiple routes will provide greater understanding of the optimal approach to induce mucosal responses.

In conclusion, these results suggest that immunization of mucosal surfaces may not be required for the generation and detection of HIV-1-specific CTL that can home to the mucosa. The application of these minimally invasive, well-tolerated procedures, such as cervical cytobrush and rectal biopsy, to assess mucosal immune responses now lend opportunity to further define the local memory responses necessary to elicit by candidate HIV-1 vaccines, and to weigh the relative importance of mucosal T cells and Abs. These investigations hold promise for enhancing vaccine efficacy in sexual transmission of HIV-1 infection.

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