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Polyclonal expansion of cervical cytobrush-derived T cells to investigate HIV-specific responses in the female genital tract

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Summary

Human immunodeficiency virus (HIV) -specific T-cell responses are detectable in the female genital tract of HIV-infected women but little is known about their frequency or the factors that influence their detection. We investigated the feasibility of polyclonal *in vitro* expansion of cervical cytobrush-derived T cells to investigate HIV-specific responses in the female genital tract in HIV-infected women. Cytobrush-derived cervical cells were isolated from 22 HIV-infected women and expanded with anti-CD3 and recombinant interleukin-2. Cervical T-cell lines were investigated for Gag-specific responses by interferon-γ ELISPOT and compared with those detected in matched blood samples. Cervical T-cell lines were established from 16/22 (72·7%) participants. Although the absolute number of $CD3^{\pm}$ cells recovered after expansion was positively associated with the number of cells isolated *ex vivo* ($P = 0{\cdot}01$; $R = 0{\cdot}62$), we observed a significant negative correlation between fold expansion and *ex vivo* cell number ($P = 0{\cdot}004$; $R = -0{\cdot}68$). We show that both the magnitude ($P = 0{\cdot}002$; $R = 0{\cdot}7$) and specific Gag regions targeted by cervical T-cell lines ($P < 0{\cdot}0001$; $R = 0{\cdot}5$) correlated significantly with those detected in blood. With one exception, cervical interferon-γ T-cell responses to Gag were detected only in HIV-infected women with blood Gag-specific response > 1000 spot-forming units/10^6 cells. We conclude that cervical Gag-specific T-cell responses in expanded lines are most easily detectable in women who have corresponding high-magnitude Gag-specific T-cell responses in blood.

Keywords: cervical; expansion; gag; human immunodeficiency virus; mucosal; T cell

Introduction

Sexual transmission of human immunodeficiency virus (HIV) accounts for the majority of new infections worldwide.[1] In women, the mucosal surface of the female genital tract is ultimately the site of HIV infection and transmission.[2] Although there is clear evidence that HIV-specific cytotoxic T lymphocytes (CTLs) in blood play an important role in controlling HIV replication systemically,[3–7] comparatively little is known about both CTL responses to HIV in the genital tract and the factors that govern these responses. HIV-specific mucosal CTLs have been detected in men and women during chronic HIV infection.[8–14] The potential importance of such CTLs in protection from HIV is highlighted by the fact that they have also been detected in individuals who, despite frequent exposure to HIV-1, fail to become productively infected.[10] In chronically HIV-infected men[14] and women,[13] the detection of HIV-specific T cells in the genital tract was independent of HIV shedding and viral load in genital secretions.

There is an urgent need for reliable, validated and non-invasive methods for investigating mucosal immune responses in the female genital tract. Such methods would be particularly valuable in HIV vaccine trial settings where intensive mucosal sampling is not an option. To date, however, the HIV-specific mucosal CTL literature has been dominated by biopsy approaches to isolating lymphocytes from rectal and gastrointestinal mucosal tissue.[9,15–19] Fewer approaches are available for sampling

mucosal tissue from the female genital tract (cervical lavages and cervical cytobrushes). The usefulness of both these methods is, however, constrained by the low numbers of *ex vivo* lymphocytes they yield.[10,12–14]

Here we investigate the feasibility of polyclonal *in vitro* expansion of cervical cytobrush-derived T cells to investigate HIV-specific responses in the female genital tract. We show that cervical T cells sampled from women with chronic HIV-1 infection can be expanded and that the magnitude of expansion is significantly associated with initial *ex vivo* cell yield and viability. Following *in vitro* expansion, we found that both the magnitude and breadth of HIV-specific T-cell responses at the cervix correlate significantly with those detected in blood. Cervical responses were, however, generally only detectable in women with corresponding blood HIV-specific T-cell responses above 1000 spot-forming units (SFU)/10^6 cells.

Materials and methods

Study population

Twenty-seven women with chronic HIV infection were enrolled. All women had CD4 counts > 300 cells/μl and were antiretroviral therapy naïve at the time of study. Samples were not collected if participants were menstruating. All women gave informed consent and the Research Ethics Committee of the University of Cape Town approved all aspects of the study.

Collection and processing of cervical and blood specimens

Cervical samples were collected using a cytobrush as previously described.[13,20] Briefly, a Digene cervical cytobrush was inserted into the cervical os and rotated through 360°. The cytobrush was immediately placed in a 15-ml tube containing ice-cold transport medium or R10 (RPMI-1640 medium, supplemented with 10% heat-inactivated human AB serum, 5 mM L-glutamine, fungazone, 50 U/ml penicillin and 50 μg/ml streptomycin). The cervical samples were kept in a Bench-top cooler (Nalgene, Rochester, NY, USA) at 4° until transport to the laboratory and were processed within 4 hr of sampling. Of the 27 samples collected, five (18·5%) were discarded because they were visibly contaminated with blood. Red blood cell contamination was measured by macroscopic visual inspection of cells in suspension and following centrifugation. We have previously demonstrated that macroscopic assessment of red blood cell contamination has a threshold of sensitivity equivalent to $\leq$ 0·0005% peripheral blood mononuclear cell (PBMC) contamination per cytobrush sample.[20]

Cervical cells were isolated from the cytobrush by flushing approximately 20 times using a sterile plastic disposable pipette to dislodge mucus. The cell suspension was centrifuged at 437 ***g*** for 10 min and the pellet was resuspended in R10.

Whole blood was collected in ACD Vacutainer tubes [Becton Dickinson (BD) Biosciences, Plymouth, UK] by venepuncture. The PBMCs were isolated from whole blood by density gradient centrifugation using Ficoll–Histopaque (Sigma-Aldrich, Egham, Runnymede, UK) and LeucoSep® centrifuge tubes (Greiner Bio-one, Frickenhausen, Germany). Cell concentrations were adjusted to 1×10^6 to 2×10^6 cells/ml and incubated overnight at 37° in 5% CO_2 for use in enzyme-linked immunosorbent spot-forming cell assays (ELISPOT). Mononuclear cells were counted using Trypan Blue staining to assess viability.

Expansion of cervical T cells

Polyclonal expansion of cervical cells was performed using anti-CD3 [Anti-CD3 monoclonal antibody (mAb); Clone UCHT1; Lot no MAB100; R&D Biosystems, Minneapolis, MN, USA] in the presence of recombinant human interleukin-2 (rhIL-2; NIH AIDS Research and Reference Reagent Program, Germantown, MD). UCHT1 is a well-characterized immunoglobulin G1 mouse mAb that identifies a determinant present on all human T cells and appears to share the same characteristics as OKT3.[21] Freshly isolated cervical cells were plated (at 4×100 μl per well per cytobrush) into 96-well round-bottomed plates pre-coated with anti-CD3 mAb (10 μg/ml). Irradiated autologous PBMCs (10^6 cells/ml; 100 μl/well; irradiated at 40 Gy) were added to each well as feeders. Recombinant hIL-2 was added to each well at a final concentration of 100 IU/ml. Cervical cytobrush T-cell lines were incubated at 37° in 5% CO_2 and cultures were supplemented every 2 days with fresh R10 containing rhIL-2 (100 IU/ml). Wells containing irradiated feeder cells alone in the presence and absence of anti-CD3 mAb and rhIL-2 were included on each plate to control for outgrowth of feeders. The T-cell lines were re-stimulated after 14 days with anti-CD3 mAb (10 μg/ml) and fresh medium supplemented with rhIL-2 (100 IU/ml). Throughout culture, all R10 was supplemented with 2 mg/ml fungazone, 50 U/ml penicillin and 50 μg/ml streptomycin and cell lines were monitored for bacterial and fungal infections, periodically counted and adjusted to 1×10^5 cells/well.

Cervical cytobrush cells and T-cell lines were analysed for expression of CD4 and CD8 T-cell markers before and after polyclonal expansion by flow cytometry. Because the number of cervical mononuclear cells available on day 0 was limited, only 10% (volume/volume) of the sample was used for determination of the CD4 : CD8 ratio on day 0. The antibodies used were allophycocyanin-conjugated CD3 (CD3-APC), fluorescein isothiocyanate-conjugated CD8 (CD8-FITC) and CD4-FITC (BD Biosciences, San Jose, CA). Cells were resuspended in 0·5 ml R10 and 50–100 μl (depending on cell count) of

cell suspension was aliquoted for surface staining. Flow cytometric acquisition was performed using a four-colour FACSCalibur flow cytometer (BD Biosciences) and analysed using FLOWJO (Tree Star, Inc., Ashland, OR) software.

HIV-1 Gag peptides

The HIV-1 subtype C Du422 Gag overlapping peptides spanning the entire Gag sequence were kindly provided by Dr Clive Gray (National Institute for Communicable Diseases, Johannesburg, South Africa) and consisted of 66 (15-mer to 18-mer) peptides overlapping by 10 amino acids. Lyophilized stocks of each peptide were dissolved in dimethyl sulphoxide at a concentration of 20 mg/ml and stored at − 80°. The 66 Gag peptides were divided into five pools each containing 14 peptides (except for pool 5, which had 10 peptides).

Interferon-γ ELISPOT

The production of interferon-γ (IFN-γ) by HIV Gag-specific T cells was detected by ELISPOT. Briefly, 96-well nitrocellulose plates were pre-coated with IFN-γ mAb (clone 1-D1K; Mabtech, Stockholm, Sweden). Cervical mononuclear cells were washed twice, counted and plated into wells either with or without HIV-1 subtype C Gag peptides (1 μg/ml) or in phytohaemagglutinin. Fresh PBMCs were plated in triplicate at 1×10^5 cells/well and expanded mucosal cells were plated at $0{\cdot}5 \times 10^5$ cells/ml. Plates were incubated overnight at 37° in a 5% CO_2 humidified incubator. After washing, bound IFN-γ was detected by a second biotinylated IFN-γ mAb (clone 7-B6-1; Mabtech), followed by streptavidin-bound horseradish peroxidase and visualization was performed using a Nova Red substrate kit (Vector Laboratories, Burlingame, CA). Finally, individual cytokine-producing cells (spots) were counted using a CTL ELISpot reader (Cellular Technology, Jessup, MD). Totals for plated wells were averaged and normalized to numbers of IFN-γ spot-forming cells per 1×10^6 PBMCs (SFU/10^6 PBMCs). Mean values for negative media control wells were subtracted from the mean values of antigen-stimulated wells to give net SFU/10^6 cells. Assays with a negative control background mean of < 100 SFU/10^6 cells were considered valid and a positive response was defined as exceeding the mean + 2 standard deviations.[12]

Intracellular cytokine staining of PBMCs following HIV Gag stimulation

To define which T-cell subset was contributing to the HIV Gag-specific IFN-γ responses detected by ELISPOT, we also assessed IFN-γ production by PBMCs using intracellular cytokine staining. The PBMCs (2×10^6 to 3×10^6 cell/ml) were stimulated with HIV Gag peptides (1 μg/ml), staphylococcal enterotoxin-B (10 μg/ml; Sigma-Aldrich; positive control) or not stimulated (negative controls) for 6 hr at 37° in 5% CO_2. Brefeldin A (10 μg/ml; Sigma, St Louis, MO) was added after the first hour. After 6 hr, cells were washed with fluorescence-activated cell sorting (FACS) wash buffer [10% fetal calf serum–phosphate-buffered saline (FCS-PBS) containing 0·01% NaN_3] and centrifuged at 200 ***g*** for 5 min. The cells were fixed and permeabilized with 2 ml BD cytofix/cytoperm solution (BD Biosciences-Pharmingen, San Diego, CA) for 10 min at room temperature. Cells were then washed once with 0·1% weight/volume Saponin (Fluka Biochemica, Mulhouse, France) with PBS (containing 5% FCS and 0·01% NaN_3) at 200 **g** for 5 min. Cells were then stained with phenotypic markers APC-labelled anti-CD3, peridinin chlorophyll protein (PerCP) -labelled CD4, and FITC-labelled anti-CD8 (BD Biosciences). To detect IFN-γ, cells were simultaneously stained with phycoerythrin (PE) -labelled anti-IFN-γ (BD Biosciences-Pharmingen) for 30 min on ice. Cells were washed with 2 ml FACS wash buffer, centrifuged at 200 **g** for 5 min and fixed with 1% paraformaldyde in PBS. Stained PBMCs were acquired on a FACSCalibur flow cytometer (BD Biosciences) and data were analysed using either CELLQUEST (BD Biosciences) version 4 or FLOWJO (Tree Star, Inc) software version 6. To define a cut-off for positive HIV Gag responses by either $CD8^+$ or $CD4^+$ T cells, IFN-γ responses to Gag peptides were initially evaluated in a panel of 10 HIV-negative women [Western blood Transfusion Service (WBTS), Cape Town, South Africa]. $CD8^+$ or $CD4^+$ T-cell responses to Gag greater than threefold above background were considered positive.

Impact of polyclonal expansion on Vβ T-cell receptor usage by PBMCs

The impact of polyclonal expansion on Vβ T-cell receptor (TCR) usage by PBMCs was evaluated using the IO-Test Beta Mark flow cytometry kit according to the manufacturer's protocol (Immunotech, Marseille, France). The kit contains eight distinct combinations of conjugated TCR Vβ antibodies corresponding to 24 different specificities (Vβ1, Vβ2, Vβ3, Vβ4, Vβ5.1, Vβ5.2, Vβ5.3, Vβ7.1, Vβ7.2, Vβ8, Vβ9, Vβ11, Vβ12, Vβ13.1, Vβ13.2, Vβ13.6, Vβ14, Vβ16, Vβ17, Vβ18, Vβ20, Vβ21.3, Vβ22, Vβ23). Briefly, unexpanded (day 0) or polyclonally expanded (day 28) PBMCs (2×10^6 to 3×10^6 cells/ml) from four individuals [patient identities (PID) 23–26] were each divided into eight BD FACS tubes. The eight tubes were then stained with a panel of 24 Vβ family-specific antibodies (three Vβ specificities/tube). Antibodies directed against Vβ specificities were restricted to FITC, PE and APC, so we added anti-CD3 PerCP (BD Biosciences, San Diego) to differentiate T-cell subsets. All

tubes were incubated at 4° for 30 min, then washed twice and fixed by the addition of 500 µl BD Cell fix. Cells were acquired using a FACSCalibur flow cytometer (BD Biosciences) and were analysed using FLOWJO (Tree Star, Inc) software.

Statistical analysis

Statistical analyses were performed using GRAPHPAD PRISM 5 (Graph Pad Software, San Diego). The Mann–Whitney *U*-test was applied for independent sample comparison, the Wilcoxon Ranks Test was used for matched non-parametric comparisons and Spearman Ranks or Pearson correlation was applied for correlation comparisons. A *P*-value of $\leq 0{\cdot}05$ was considered significant.

Results

Twenty-two women with chronic HIV infection were investigated for HIV Gag-specific cervical cytobrush-derived T-cell responses following *in vitro* expansion (Table 1). All the study subjects were under the age of 40 years (median age 31 years, range 22–39), had a median blood CD4 count of 451 cells/µl (range 300–1300) and plasma viral load of 10 097 (range 970–1 300 000). Five of the 22 (22·7%) women had visible vaginal discharge or yeast infections at the time the cytobrush was taken.

Expansion of cervical cytobrush-derived T cells from women with chronic HIV infection

We obtained a median of $0{\cdot}1 \times 10^6$ cells (range $0{\cdot}01 \times 10^6$ to $0{\cdot}2 \times 10^6$ cells) per cytobrush *ex vivo* (Table 1). We initially investigated the association between *ex vivo* cervical cell yields and both age and clinical HIV disease status (CD4 counts and viral load). We observed a significant positive association between the age of women in the study and the yield of cervical cells obtained ($P = 0{\cdot}0008$; Pearson $R = 0{\cdot}66$) indicating that older age was associated with increased yield of cervical cells. We found no association between markers of disease status (CD4 count and viral load) and cervical yield (data not shown). However, all women included in this study were recruited during the chronic phase of infection with CD4 counts $\geq$ 300 cell/µl (CD4 counts ranging from 300

Table 1. Description of human immunodeficiency virus (HIV) -infected women included in the study

Patient ID	Age	CD4 count (cell/µl)	HIV viral load (RNA copies/ml)	Macroscopic findings	Day 0 cervical cell counts	Day 14 cervical cell counts	Day 28 cervical cell counts	Fold expansion (day 28)
1	33	1396	< 50[1]	Vaginal discharge	20 000	100 000	1 600 000	80
2	26	414	9500	Nil	10 000	100 000	680 000	68
3	23	341	14 500	Nil	10 000	310 000	480 000	48
4	30	428	< 50[1]	Nil	14 278	Contaminated	Contaminated	–
5	28	698	1800	Vaginal discharge	18 520	Contaminated	Contaminated	–
6	28	341	100 573	Nil	40 000	130 000	560 000	14
7	36	572	51 325	Nil	68 640	125 600	1 650 000	24
8	26	607	4013	Nil	75 800	114 000	Contaminated	–
9	31	482	27 250	Nil	80 000	140 000	990 890	12·4
10	30	545	15 800	Nil	90 000	440 000	1 780 000	19·8
11	32	400	1 300 000	Cervix inflamed	90 860	Contaminated	Contaminated	–
12	31	393	41 700	Nil	100 000	610 000	850 000	8·5
13	22	301	2200	Nil	100 000	270 000	1 750 000	17·5
14	32	461	7100	Nil	110 000	490 000	1 040 000	9·5
15	31	788	1926	Nil	120 000	790 000	1 200 000	10
16	33	451	30 000	Nil	120 000	260 000	1 900 000	15·8
17	24	ND	3649	Nil	135 721	617 600	1 460 000	10·8
18	39	422	5450	Nil	138 040	652 000	Contaminated	–
19	39	338	< 50[1]	Nil	180 000	240 000	1 200 000	6·7
20	43	467	88 000	Yeast	218 064	Contaminated	Contaminated	–
21	37	300	10 097	Vaginal discharge	220 000	740 000	2 120 000	9·6
22	38	482	970	Nil	240 000	530 000	2 850 000	11·9
Median	31	451	10 097	5/22 (22·7%)	100 000	290 000	1 330 000	13·2
Range	22–39	300–1300	970–1 300 000		10 000–240 000	132 500–590 000	480 000–2 850 000	6·0–80·0

[1]Less than detectable Limit.

to 1300 cells/μl; Table 1). Age and clinical status were not associated with viability of cervical cytobrush cells in this study (data not shown). None of the women in the study were menstruating at the time of cytobrush collection although we did not control for menstrual cycle stage. We have previously shown that *ex vivo* cervical cytobrush mononuclear cell yields are significantly associated with genital tract inflammation.[22] Although we have not measured inflammatory cytokine concentrations in genital secretions in this study, genital inflammatory mediators are likely to have a substantial impact on *ex vivo* cervical mononuclear cell yield.

Cervical T cells were polyclonally expanded for either 28 days (18/22) or 42 days (4/22) to increase the yield of cells from the cervix for the identification of peptide responses to Gag by IFN-γ ELISPOT. Of the 22 cervical T-cell lines initiated, 16/22 (72·7%) expanded. We obtained a median of $1{\cdot}3 \times 10^6$ cells (range $0{\cdot}5 \times 10^6$ to $2{\cdot}9 \times 10^6$ cells) at day 28, which represented a 13-fold increase in T-cell yield compared with *ex vivo* cell yields (Table 1 and Fig. 1a). Six of 22 lines (27·3%) were lost to contamination during the first few days of culture. Of these, three of the six contaminated samples were collected from women with visible cervical discharge.

The CD8 T cells were found to be the dominant T-cell subset detected *ex vivo* (CD4 : CD8 of 0·5); however, 28 days of *in vitro* expansion resulted in the ratio equalizing such that we obtained a CD4 : CD8 ratio of 1 (Fig. 1b; $P = 0{\cdot}006$, Wilcoxon Rank Test). Of the cervical

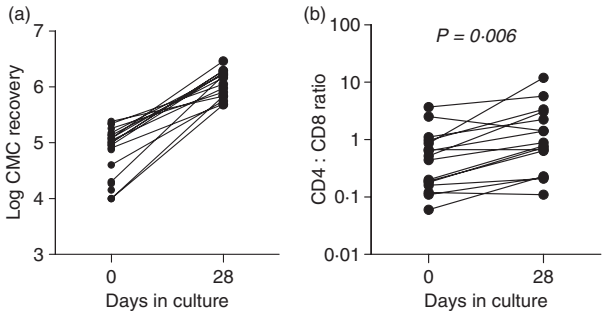

Figure 1. Impact of *ex vivo* yield on expansion of cervical cytobrush-derived cervical mononuclear cells (CMC). (a) Comparison between day 0 and day 28 CMC yields in donors whose cervical CD3 cells expanded. (b) Comparison of CD4 : CD8 ratio *ex vivo* (day 0) and day 28 in expanders. CMC were cultured *in vitro* with anti-CD3 monoclonal antibody in the presence of recombinant human interleukin-2 (rhIL-2). The CMC counts and viability were performed by Trypan blue counting. CD4 : CD8 ratios on cervical cell lines were analysed before and after polyclonal expansion using allophycocyanin conjugated CD3 and fluorescein isothiocyanate-conjugated CD8 and CD4 staining on a FACSCalibur flow cytometer and analysis was performed using FLOWJO software. Wilcoxon Rank Test was applied to compare yield and CD4 : CD8 ratios at day 0 and day 28. *P*-values < 0·05 were considered significant. Each data point represents an individual donor.

lymphocytes isolated *ex vivo*, 90·9% (± 7·5; mean ± SD) were viable (range 70–100%). We found a significant positive correlation between the absolute number of cells obtained following expansion and both the number of cervical cells isolated *ex vivo* ($P = 0{\cdot}01$; $R = 0{\cdot}62$) and the *ex vivo* viability of cells ($P = 0{\cdot}0002$; $R = 0{\cdot}71$). Conversely, we found a significant negative correlation between fold expansion by 28 days and *ex vivo* cell number ($P = 0{\cdot}004$; $R = -0{\cdot}68$), indicating that *ex vivo* cervical cells with the lowest yield exhibited the highest relative rate of expansion.

Gag-specific responses by cervical T cells

The HIV-1 Gag-specific responses were mapped by IFN-γ ELISPOT for 16 of 22 cervical T-cell lines (72·7%) from chronically HIV-infected women. These were compared with similarly identified responses identified in PBMCs *ex vivo* of these women (Fig. 2). Nine of the 16 (56·2%) cervical lines from the chronically HIV-infected women showed clear evidence of HIV-specific T-cell responses to Gag with a cumulative frequency of IFN-γ-producing cells ranging from 350 to 13 553 SFU/10^6 cells. We found no correlation between cervical *ex vivo* yield, viability, expansion (day 28) yield, or plasma viral load and the magnitudes of HIV Gag-specific cervical T-cell responses (data not shown). Eight of the nine cervical lines that exhibited Gag specificity were derived from women with clearly detectable Gag-specific blood responses (>600 SFU/10^6 cells; Fig. 2). There was only one participant who had a detectable cervical response to Gag but no matching blood response.

From the 16 women for which HIV Gag ELISPOT mapping was performed (Fig. 2), there was a significant correlation between the cervix and blood in both the total magnitude of IFN-γ responses to Gag (Table 2 and Fig. 3a; rho = 0·7, $P = 0{\cdot}002$; Spearman Ranks test) and the individual Gag pool magnitudes of IFN-γ responses per individual (Table 2 and Fig. 3b; rho = 0·51, $P < 0{\cdot}0001$; Spearman Ranks test). Despite good maintenance of specific Gag pool targeting between cervix and blood, four of the nine women (44·4%) with detectable HIV-specific responses at the cervix had unique pool specificities that were not detected in blood (PID 1, 2, 17 and 19; Fig. 2 and Table 2).

Individual peptide mapping was performed on cervical lines derived from four of the nine women with detectable Gag pool responses who had sufficient cervical cell numbers to allow finer mapping (PID 2, 7, 15 and 19; Table 3). All four women had cervical T-cell responses that preferentially targeted peptides in p24 and three of the women targeted the same peptide within p24 containing the human leucocyte antigen (HLA) class I TL9 epitope (^{178}GA**TPQDLNTML**NTVGGH$_{194}$). Although the HLA for the participants in this study are not known, the

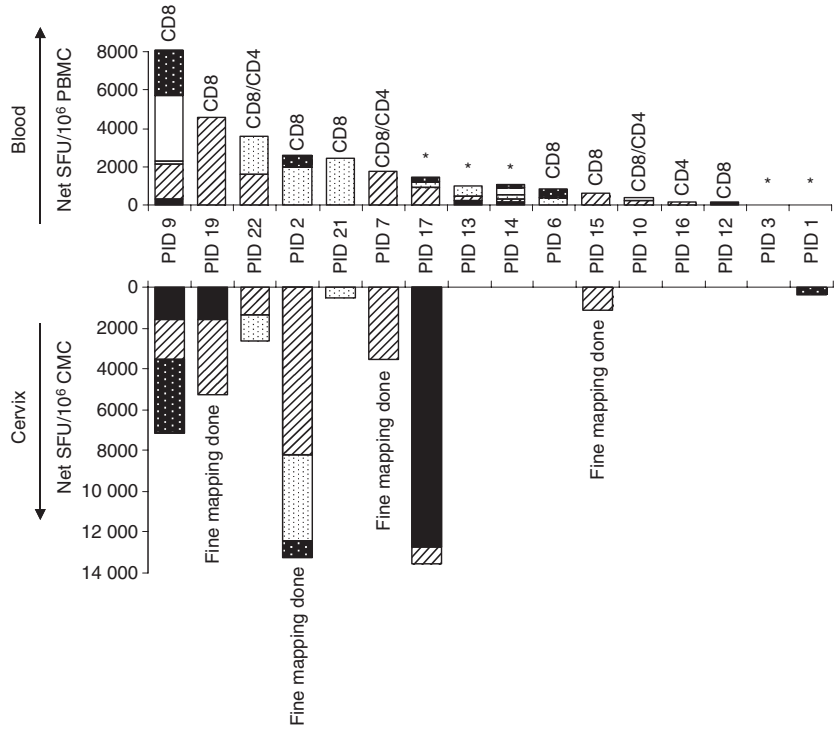

Figure 2. Comparison of cervical and blood human immunodeficiency virus (HIV) Gag-specific interferon-γ (IFN-γ) responses in women with chronic HIV infection. Blood (top panel) and cervical (bottom panel) IFN-γ responses to HIV-1 subtype C Gag peptide pools 1–5. Net spot forming units (SFU)/10^6 was calculated by subtracting background IFN-γ frequency from Gag peptide pool-specific IFN-γ frequencies in 16 women with chronic HIV infection. Each stacked bar represents the cumulative IFN-γ frequency for all five Gag peptide pools and the subjects have been ordered from highest to lowest blood T-cell magnitude to Gag. The contribution of $CD4^+$ and $CD8^+$ T-cell subsets to overall IFN-γ responses to HIV Gag peptides was determined by intracellular cytokine staining and fluorescence-activated cell sorting. The T-cell subset contributing to Gag-specific responses per donor is shown above each stacked bar for blood responses. CD8 indicates CD8-only response, CD4 indicates CD4 responses only and CD8/CD4 indicates that responses in both subsets were detected. Asterisks indicate that responses were not evaluated in these individuals. 'Fine mapping done' denotes the four women for whom this analysis was carried out.

peptides recognized by these four women had a number of previously characterized HLA class I and fewer HLA class II epitopes embedded within them (Table 3; predicted by the Los Alamos Database; http://www.hiv.lanl.gov/content/sequence/ELF/epitope_analyzer.html).

Although the low cervical cell numbers generated in this study did not allow us to evaluate whether the cervical Gag-specific responses detected (Fig. 2) were specific to the $CD8^+$ or $CD4^+$ T-cell subsets, we evaluated T-cell subset-specific IFN-γ responses to Gag in matched blood samples from 11/16 women by intracellular cytokine staining. The majority of these women had only $CD8^+$ T-cell responses to Gag (7/11; PID 2, 6, 9, 12, 15, 19, 21), three women had Gag-specific responses in both $CD4^+$ and $CD8^+$ subsets (PID 7, 10, 22) and one woman had only a $CD4^+$ response (PID 16). Of these 11 women, seven had detectable cervical responses to Gag. Five of these seven women had exclusively $CD8^+$ T-cell responses to Gag in blood and two had both $CD4^+$ and $CD8^+$ T-cell responses.

Although it was not possible to confirm which T-cell subset was contributing to the cervical T-cell responses detected in this study (Fig. 2), the fact that the individual peptides mapped in four individuals (Table 3) contained both HLA class I and II epitopes and that flow cytometry on matched blood samples showed both $CD8^+$ and $CD4^+$ responses systemically to Gag implies that both T-cell subsets may be involved. The dominance of $CD8^+$ responses in blood together with the number of HLA class I predicted epitopes suggests, however, that $CD8^+$ T-cells are likely to be the dominant T-cell subset involved in the female genital tract as well.

Impact of expansion on HIV-specificity and T-cell Vβ repertoire in blood

To evaluate the impact of polyclonal expansion with anti-CD3 mAb on skewing of Gag regions targeted by HIV-specific T cells, PBMCs from four HIV-infected individuals with well-characterized *ex vivo* Gag-specific responses were examined before and following expansion by IFN-γ ELISPOT (Fig. 4). The dominant *ex vivo* Gag regions targeted were maintained following *in vitro* expansion in all individuals. Compared with *ex vivo* responses (day 0), there

Table 2. Comparison between breadth of response at cervix and in blood

Patient ID	Cumulative magnitude (spot-forming units/10^6)		Gag pools shared	Gag pools differently targeted	
	Blood	Cervix		Blood	Cervix
9	8040	7130	1,2,5	3,4	–
19	4605	5280	2	–	1
22	3565	2640	2,3	–	–
2	2610	13 240	3,5	–	2
21	2440	490	3	–	–
7	1740	3540	2	–	–
17	1480	13 553	2	3,5	1
13	1000	0	–	1,2,3	–
14	1100	0	–	1,2,3,4,5	–
6	850	0	–	3,5	–
15	600	1140	–	2	–
10	360	0	–	2,4	–
16	0	0	–	2	–
12	165	0	–	1	–
3	120	0	–	–	–
1	0	350	–	–	5
Median	1050	420			
Range	0–8040	0–13 553			

was an overall increase in the cumulative frequency of IFN-γ-producing cells following *in vitro* expansion, ranging from twofold to 11-fold. The frequency of Gag pool responses at day 0 was, however, significantly correlated with the frequencies following expansion for 28 days (Fig. 4b; $R = 0{\cdot}81$; $P < 0{\cdot}0001$; Spearman Ranks Test) indicating that the specificity profile was largely maintained following expansion. In a single donor (PID 26), however, a unique specificity to pool 5 (485 SFU/10^6 cells) was observed after expansion that was not detectable *ex vivo*. This highlights the possibility that specificities of low frequency that are not apparent *ex vivo* may become detectable following expansion because of the observed overall increase in cumulative frequency. It is also important to note that only one of the four unique Gag specificities that we observed in cervical samples (PID 1) had a magnitude of < 500 SFU/10^6 cells whereas unique cervical specificities from the other three donors (PID 2, 17, 19) had magnitudes of > 1500 SFU/10^6 cells. This implies that the unique cervical specificities we recorded for at least three of the four cervical donors represent 'real' unique responses and are not the result of expansion of previously low-frequency events.

We next evaluated changes in the TCR Vβ repertoire following 28-day expansion of PBMCs with anti-CD3 and rhIL-2 in these four individuals (Table 4). The profile of TCR Vβ frequencies on day 0 in three of the four study subjects correlated significantly with profiles following *in vitro* polyclonal expansion (patient 26: $R = 0{\cdot}97$, $P < 0{\cdot}0001$; patient 24: $R = 0{\cdot}69$, $P = 0{\cdot}0002$, patient : $R = 0{\cdot}84$, $P < 0{\cdot}0001$). In contrast, TCR Vβ frequencies for patient 23 following expansion did not correlate and this was probably as a result of the expansion of certain Vβ types. In this individual, Vβ4 expanded 9·9-fold, Vβ7.2 expanded 4·6-fold and Vβ13.1 contracted 7·6-fold over 28 days. We were unable to similarly compare fluctuations in cervical TCR Vβ specificities during the course of expansion because of limiting cervical cell numbers.

Discussion

Immunological events at the cervix are likely to influence susceptibility to heterosexually-transmitted HIV infection.

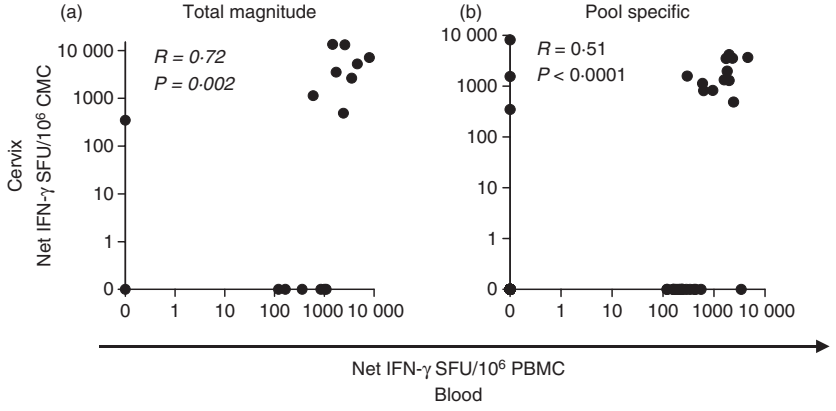

Figure 3. Correlation between human immunodeficiency virus (HIV) Gag-specific interferon-γ (IFN-γ) response magnitudes detected at the cervix and in blood of HIV-infected subjects. (a) Correlation between cumulative net IFN-γ responses to Gag pools (sum of five pools) per chronically HIV-infected individual in each compartment ($n = 16$ women for whom matched enzyme-linked immunosorbent spot-forming cell assay was conducted on cervical lines and peripheral blood mononuclear cells). (b) Correlation between individual Gag pools (1–5) at the cervix and in blood in HIV-infected women. Each data point represents an individual's IFN-γ response at the cervix and in blood. Spearman Rank Test was applied to test correlations and P-values < 0·05 were considered significant. Spearman R-value is shown on each plot.

Table 3. Epitope prediction from confirmed peptide specificities on cervical samples

PID	HIV protein	Net SFU/ million cells	Peptide	HLA I epitope	HLA I type	HLA II epitope	HLA II type
19	p24	880	24	^{170}PMFT**ALSEGATPQDL**NTM$_{187}$		^{170}**PMFTALSEGAT**PQDLNTM$_{187}$	
				^{170}PMFTA**LSEGATPQDL**NTM$_{187}$	B42, B*4403		
	p24	4680	25	^{178}GA**TPQDLNTML**NTVGGH$_{194}$	B*3910, B*4201, B*8101, B*0702, Cw*0802	^{178}GA**TPQDLNTMLNTVGGH**$_{194}$	
2	p24	5600	22	^{155}W**VKVIEEKAF**SPEVIPMF^{172}	B*1503	^{155}WVKVI**EEKAFSPEV**IPMF^{172}	DRB1*0101
				^{155}WVKV**IEEKAFSPEV**IPMF^{172}	B*4006	^{155}WVKVI**EEKAFSPEVIP**MF^{172}	DQ5
				^{155}WVKV**IEEKAFSPEVI**PMF^{172}	B*4501		
				^{155}WVKVI**EEKAFSPEV**IPMF^{172}	B*4501		
				^{155}WVKVIE**EKAFSPEV**IPMF^{172}	Cw*0602		
	p24	2040	25	^{155}WVKVIEE**KAFSPEVIPMF**^{172}	B57, B63	^{178}GA**TPQDLNTMLNTVGGH**$_{194}$	
	p24	6120	33	^{155}WVKVIEEKA**FSPEVIPMF**^{172}	B63, B57, B58	^{234}SDIA**GTTSTLQEQIA**WM^{250}	
	p2p7p1p6	680	57	^{155}WVKVIEEKAF**SPEVIPMF**^{172}	B*35		
				^{178}GA**TPQDLNTML**NTVGGH$_{194}$	B*3910, B*4201, B*8101, B*0702, Cw*0802		
				^{234}SDIAGT**TSTLQEQIAW**M^{250}	B*5701, B*5703, B*5801		
				^{414}W**KCGKEGHQMK**DCTERQA^{431}	A11		
15	p24	3210	25	^{178}GA**TPQDLNTML**NTVGGH$_{194}$	B*3910, B*4201, B*8101, B*0702, Cw*0802	^{178}GA**TPQDLNTMLNTVGGH**$_{194}$	
7	p24	3820	23	^{163}**AFSPEVIPMFT**ALSEGA^{179}		^{163}AF**SPEVIPMFTALSE**GA^{179}	
				^{163}AF**SPEVIPMF**TALSEGA^{179}	B63, B57, B58		
				^{163}AFSP**EVIPMFTAL**SEGA^{179}	A*2601, A*2603		

HLA, human leucocyte antigen; HIV, human immunodeficiency virus; PID, patient identity; SFU, spot-forming units. Previously defined immunodominant epitope are represented in bold.

There is an urgent need for the development of validated, non-invasive methodologies for investigating HIV-specific mucosal immune responses associated with HIV pathogenesis and for vaccine assessment specifically in the female genital tract.[10,17] Although cervical cytobrushing is relatively non-invasive, low cervical cell yields associated with this approach have significantly impacted on thorough evaluation of HIV-specific T-cell responses.[10,13,17] Although *in vitro* expansion of cervical cytobrush-derived T cells would circumvent the problem of low yield, there have been few studies investigating the ability of cervical cytobrush-derived T cells to expand *in vitro* either polyclonally or in response to HIV-1 antigens. We show here that cervical T cells can be isolated by cytobrushing and polyclonally expanded in the majority of women studied. Further, we show that HIV Gag-specific cervical T-cell responses can be detected in HIV-infected women and correlate with responses detected in blood.

With one exception, cervical responses to Gag were detected only in HIV-infected women with blood Gag-specific responses > 1000 SFU/10^6. Although we achieved a 13-fold expansion of cervical cell numbers, our inability to resolve genital tract responses to Gag in individuals with < 1000 SFU/10^6 cells in blood suggests that our approach may not be sensitive enough for the evaluation of vaccine efficacy. The reason for this is that vaccine induced T-cell responses to Gag are expected to be substantially lower than those found in HIV-infected individuals. Although the bottleneck imposed by non-invasive cytobrush sampling impinges on the sensitivity achievable in cellular assays such as those described in this study, it is likely that a combination of even moderately improved sampling and cellular expansion methodologies could substantially lower the threshold above which HIV-specific cellular responses could be detected.

Cervical cytobrush-derived T cells were polyclonally expanded *in vitro* from 73% of samples analysed. We achieved a median of 13-fold expansion of cervical mononuclear cells using anti-CD3 over 28 days. Ibarrondo *et al.*[9] similarly expanded rectal biopsy-derived mucosal T cells from 12 HIV-infected individuals and reported a fourfold to 10-fold expansion of $CD8^+$ T cells over 14 days with bi-specific CD4/3 antibodies. Whereas their method selectively expanded $CD8^+$ T cells, anti-CD3 expansion used in the present study expanded both CD4 and CD8 T cells with an overall twofold enrichment of $CD4^+$ cells following expansion. Although the overall relative expansion described here was higher than in previous studies,[9] rectal biopsy mucosal sampling yielded 16-fold to 39-fold more cells than were obtained in our study

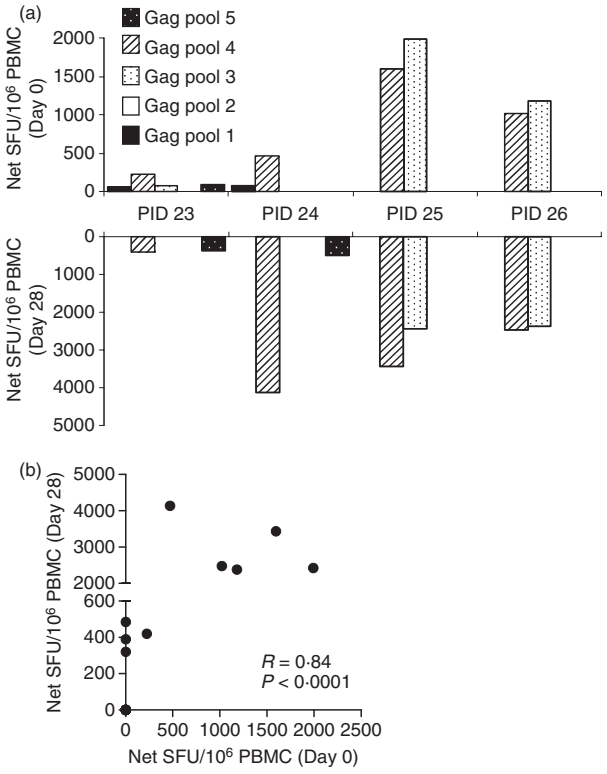

Figure 4. Correlation between *ex vivo* and expanded human immunodeficiency virus (HIV) Gag-specific interferon-γ (IFN-γ) enzyme-linked immunosorbent spot-forming cell assay responses in blood from chronically HIV-infected subjects. (a) Peripheral blood mononuclear cells (PBMCs) from four HIV-infected women were assessed for HIV-specific IFN-γ production *ex vivo* (top panel) and following *in vitro* expansion with anti-CD3 monoclonal antibody for 28 days (bottom panel). (b) Correlation between IFN-γ responses detected in PBMCs *ex vivo* and after 28 days expansion. HIV-1 subtype C Gag overlapping peptides were divided into five pools and responses in these donors to each Gag pool were assessed. Net IFN-γ response to Gag was calculated by subtracting background IFN-γ production in each individual from Gag-specific responses. Each dot (b) represents an individual donor's matched IFN-γ response to each of the five Gag pools in blood *ex vivo* and following expansion (day 28). Spearman Rank Test was used to test the correlation and a *P*-value <0·05 were considered significant.

from a single cytobrush. Although we have focused on a single expansion protocol with anti-CD3 mAb and rhIL-2, it will be important in future studies to compare cervical T-cell expansion kinetics using different expansion protocols, such as bi-specific antibodies,[23] anti-CD3/28-coated beads (T-cell expander; Dynabeads; Invitrogen, Oslo, Norway); and addition of alternative or complementary cytokines such as IL-7 and IL-15.[24,25]

In this study, 27% of cytobrush-derived cervical cultures that were initiated became contaminated within the first 24 hr of culture. Of these cytobrush samples, half were from women with macroscopic evidence of vaginal discharge, cervical inflammation or yeast infection indicating that concomitant cervical infections impact on the sterility of the sample for culture. Although our culture medium for transport of these samples to the laboratory and for processing contains penicillin, streptomycin and amphotericin B, these contaminations could possibly be minimized by including higher concentrations of amphotericin B and antibacterial agents specific for genital tract bacterial infections.

From expanded cervical T-cell lines, we found that HIV Gag-specific cervical T-cell responses were detectable in $\sim$ 50% of women with chronic HIV infection. From matched blood samples, 88% of these women had systemic responses to Gag. The magnitude of these mucosal responses correlates significantly with the magnitude of Gag-specific responses measured in blood and was largely restricted to women with > 1000 Gag-responsive cells/10^6 cells. Similarly, Kaul *et al.*[11] showed concordance between HIV-specific responses in blood and at the cervix *ex vivo* during chronic infection with 8/10 women studied having matching responses at the cervix and in blood. Kaul *et al.*[11] selected participants based on high response frequencies to Gag in blood, which may have impacted on the strong compartmental overlap they described. In comparison, Shacklett *et al.*[12] reported that only three of eight chronically HIV-infected women with detectable HIV-specific responses in blood had a corresponding response at the cervix. Shacklett *et al.*[12] also focused their study on women with well-characterized high-frequency responses in blood. In contrast to these studies, Kaul *et al.*[10] found in both HIV-infected and apparently HIV-resistant uninfected sex workers that *ex vivo* HIV-specific responses at the cervix were as prevalent as they were in blood, that responses in the two compartments largely correlated and that 95% of women with detectable HIV-specific cervical T-cell responses (40/42) had corresponding blood responses of < 1000 SFU/million cells.

Recently, our laboratory[13] reported that *ex vivo* frequencies of HIV Gag-specific CD8 responses in blood and at the cervix did not correlate. Unlike Kaul *et al.*[11] and Shacklett *et al.*,[12] our previous study[13] did not select women with high-magnitude responses in blood and this may have impacted on the lack of concordance observed between compartments *ex vivo*. Our previous study[13] focused *ex vivo* functional assessment rather than *in vitro* expansion used in the present study. *Ex vivo* cytobrush-derived T-cell effector cell distribution is likely to differ substantially from phenotypes present after *in vitro* expansion, with the former being dominated by effector cells and the latter being dominated by expanded memory cells.[26] Although we did not compare the maturational status of *ex vivo* or expanded cervical T cells, this may play a role in the success of *in vitro* expansion. Naïve and memory $CD8^+$ T-cell subsets in humans have significantly different capacities to proliferate and differentiate in response to T-cell receptor stimulation or cytokines.[27]

Table 4. T-cell receptor (TCR) Vβ usage *ex vivo* and following 28 days of *in vitro* expansion with anti-CD3 and recombinant human interleukin-2

	PID 23			PID 24			PID 25			PID 26		
TCR Usage	Day 0	Day 28	Ratio Day 28 : 0	Day 0	Day 28	Ratio Day 28 : 0	Day 0	Day 28	Ratio Day 28 : 0	Day 0	Day 28	Ratio Day 28 : 0
Vβ1	4·27	2·2	0·52	5·84	5·61	0·96	3·65	5·94	1·63	6·93	6·56	0·95
Vβ2	3·02	0·78	0·26	6·69	5·55	0·83	3·56	6·59	1·85	3·38	5·21	1·54
Vβ3	5·52	6·34	1·15	4·17	5·14	1·23	1·64	2·27	1·38	4·48	5·73	1·28
Vβ4	0·64	6·24	**9·75**[1]	1·51	3·41	2·26	1·26	1·72	1·37	0·85	0·66	0·78
Vβ5.1	3·22	3·52	1·09	3·85	4·26	1·11	3·12	6·19	1·98	4·5	5·71	1·27
Vβ5.2	1·44	1·43	0·99	0·79	0·91	1·15	1·3	1·59	1·22	1·8	1·56	0·87
Vβ5.3	2·19	0·96	0·44	1·45	1·44	0·99	1·43	1·32	0·92	0·92	1·09	1·18
Vβ7.1	3·42	3·23	0·94	2·23	2·41	1·08	2·17	3·93	1·81	1·73	2·56	1·48
Vβ7.2	0·97	4·47	4·61	1·36	1	0·74	0·66	1·56	2·36	0·34	0·85	2·50
Vβ8	3·86	4·54	1·18	3·96	4·43	1·12	4·24	4·67	1·10	4·6	4·99	1·08
Vβ9	3·4	1·69	0·50	2·18	2·58	1·18	3·56	2·37	0·67	5·09	2·52	0·50
Vβ11	0·95	1·54	1·62	1·06	1·33	1·25	1·07	1·32	1·23	0·4	1·22	3·05
Vβ12	2·17	0·82	0·38	1·47	1·4	0·95	2·17	3·02	1·39	1·53	3·57	2·33
Vβ13.1	6·15	0·81	0·13	2·96	3·69	1·25	3·2	4·82	1·51	4·27	4·98	1·17
Vβ13.2	1·3	1·41	1·08	2·62	2·61	1·00	1·24	1·59	1·28	0·97	1·66	1·71
Vβ13.6	1·97	3·03	1·54	2·31	2·43	1·05	1·74	3·19	1·83	0·89	2·3	2·58
Vβ14	2·72	2·73	1·00	10·55	11·1	1·05	6·27	4·24	0·68	3·15	3·52	1·12
Vβ16	2·36	2·39	1·01	0·93	0·95	1·02	1·59	3·18	2·00	1·27	2·4	1·89
Vβ17	2·54	4·06	1·60	3·57	3·8	1·06	2·47	6·23	2·52	5·96	4·39	0·74
Vβ18	0·64	0·55	0·86	0·36	0·46	1·28	0·56	0·62	1·11	0·81	0·96	1·19
Vβ20	3·03	3·37	1·11	2	2·41	1·21	2·61	3·48	1·33	2·38	3·19	1·34
Vβ21.3	5·42	5·48	1·01	0·55	0·87	1·58	2·64	4·42	1·67	1·99	4·17	2·10
Vβ22	2·55	5·16	2·02	3·87	3·94	1·02	2·43	4·68	1·93	3·54	3·94	1·11
Vβ23	2	4·59	2·30	0·64	0·56	0·88	0·82	1·18	1·44	0·94	1·25	1·33
Mean			1·55			1·14			1·51			1·46
Pearson *R*-value			0·1820[2]			0·9736			0·6896			0·8408
Pearson *P*-value			0·3946			< 0·0001			0·0002			< 0·0001

[1]Changes in any Vβ subset from day 28 to day 0 that were > 2 are denoted in bold.
[2]The correlation between Vβ TCR usage at day 0 and day 28 was tested using a Pearson test and *R*-values and *P*-values are shown.

There are a number of issues that may also impact on the extent of *in vitro* expansion of cytobrush-derived T cells including the stage of the menstrual cycle at which cytobrushes were collected, concomitant sexually transmitted infections, and vaginal hygiene practices.[28–30]

Although *in vitro* polyclonal expansion of T cells is a useful approach to increase the number of cells available from inaccessible mucosal sites, antigen bias and epitope skewing introduced by polyclonal expansion (potentially favouring the outgrowth of certain populations at the expense of others) cannot be entirely excluded. We show here that both $CD4^+$ and $CD8^+$ cervical T cells were expanded and that similarly expanding PBMCs did not significantly alter HIV Gag targeting nor TCR Vβ repertoire in most individuals. Some fluctuations were noted in TCR usage and the range of HIV Gag pools targeted in a minority of women, so we acknowledge that some bias may be introduced by expansion in a subset of individuals.

In summary, this study evaluates the feasibility and efficacy of polyclonal *in vitro* expansion of cervical cytobrush-derived T cells from women with HIV infection. The low cell yields that can be recovered non-invasively from the female genital tract necessitate focused efforts to optimize effective expansion methodologies. We do not fully understand what would constitute protective immunity against HIV, and immunity at the genital mucosa is likely to play an important role in preventing HIV acquisition, so the incorporation of mucosal sampling at the female genital tract and understanding HIV-specific events at this site should be a high priority during HIV vaccine trials. Our finding that cervical cytobrushing and anti-CD3-mediated polyclonal expansion only enable the detection of Gag-specific T-cell responses in the genital tracts of women with correspondingly high systemic Gag-specific responses sets a benchmark against which to measure future efforts in this field and highlights the need for more efficient expansion methodologies.

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Disclosures

None.

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