Video Article

Collection, Isolation, and Flow Cytometric Analysis of Human Endocervical Samples

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Abstract

Despite the public health importance of mucosal pathogens (including HIV), relatively little is known about mucosal immunity, particularly at the female genital tract (FGT). Because heterosexual transmission now represents the dominant mechanism of HIV transmission, and given the continual spread of sexually transmitted infections (STIs), it is critical to understand the interplay between host and pathogen at the genital mucosa. The substantial gaps in knowledge around FGT immunity are partially due to the difficulty in successfully collecting and processing mucosal samples. In order to facilitate studies with sufficient sample size, collection techniques must be minimally invasive and efficient. To this end, a protocol for the collection of cervical cytobrush samples and subsequent isolation of cervical mononuclear cells (CMC) has been optimized. Using *ex vivo* flow cytometry-based immunophenotyping, it is possible to accurately and reliably quantify CMC lymphocyte/monocyte population frequencies and phenotypes. This technique can be coupled with the collection of cervical-vaginal lavage (CVL), which contains soluble immune mediators including cytokines, chemokines and anti-proteases, all of which can be used to determine the anti- or pro-inflammatory environment in the vagina.

Video Link

The video component of this article can be found at https://www.jove.com/video/51906/

Introduction

The majority of new HIV infections worldwide arise through heterosexual transmission, with women representing 47% of new infections in 2011 (UNAIDS[1]). Understanding the female genital tract (FGT), one of the main entry portals for HIV and other sexually transmitted pathogens, is of high importance on the path to finding efficient strategies to prevent infection. Immune responses at the genital mucosa are clearly unique and differ from those measured in peripheral blood[2]. However, current knowledge of the immune dynamics at the FGT is limited at best. To date, studies of the mucosal immune environment have largely focused on the gut-associated lymphoid tissues (GALT), where it has become clear that the early events in mucosal tissues following infection have a strong impact on subsequent disease progression[3,4]. Collecting samples from the genital mucosa represents a great challenge and is at least partially responsible for the lack of understanding of the immunology of the FGT. Solving the puzzle of the immune dynamic between host and pathogen in the context of the distinct environment that is the FGT necessitates efficient methods for collecting and analyzing samples from this locale.

The FGT is divided into two sections: the upper reproductive tract that includes the fallopian tubes, endometrium and endocervix, and the lower tract which contains the ectocervix and the vagina (reviewed by Kaushic *et al*[5]). It is still unclear what the relative contribution of these different sites is to HIV infection, but it is believed that both sites could contribute to HIV entry[6]. T cells represent 40-50% of the leucocytes in the upper and lower reproductive tracts, while macrophages comprise approximately 10% (reviewed in Rodriguez-Garcia *et al*[2]). T cells can be detected in the vagina, cervix, and endometrium. Macrophages are more strongly localized in the endometrium and myometrial connective tissue than the cervix, although they can be detected in both tissues. Finally, plasmacytoid dendritic cells (pDCs) and Langerhans cells can also be detected in FGT tissues. The phenotype and proportions of immune populations and their susceptibility to HIV infection may vary importantly according to hormonal cycles, the use of hormonal contraceptives, bacterial vaginosis or sexual activities[5,7-9].

Diverse methods have been developed to study the immune populations and environment of the FGT. Cervical biopsy, cervical cytobrushes and cervicovaginal lavages (CVL)[10-12] are the most commonly used across the literature. CVL collection by PBS lavage is the simplest method and allows the study of immune modulatory proteins but results in extremely low cell yield, and is therefore not suitable for studying the immune cell populations of the FGT[13]. CVL samples are, on the other hand, very useful for evaluating the immune environment of the FGT by measuring the expression of various cytokines, chemokines or antimicrobial factors using methods such as ELISA, cytokine bead array[14] or mass spectrometry[15,16]. Characterization of immune cell frequencies, phenotypes and functions can be achieved by collecting cervical mononuclear cells (CMC) by cervical cytobrush or by cervical biopsy sampling.

Cervical biopsy sampling is an invasive method that increases the discomfort and risk of bleeding and takes 2 to 11 days to heal following the procedure depending on the immune status of the woman[12]. On the other hand, cervical cytobrushes, despite the lower yield of cells collected, is a less invasive and more convenient method to collect immune cells from the FGT. Both methods can reach the same yield of CD45+ leucocytes, but two sequential cervical cytobrushes are necessary to obtain the same amount of cells contained in one biopsy[13]. Nonetheless, cytobrush sampling still provides an acceptable number of cells (about 5,000 CD45+ cells/cytobrush) for further *ex vivo* phenotyping by flow cytometry[14]. Also, functional characterization can be carried out on these samples, as stimulation and intracellular flow cytometry or qPCR have been performed using cytobrush-derived CMCs to identify HIV-specific immune responses[17] or Th cell polarization[18]. Expansion of the T cells population may also facilitate functional studies with CMCs[19].

It is important to note that biopsies and cytobrushes sample distinct portions of the FGT. Biopsies are derived from the superior portion of the epithelium and stroma of the ectocervix[12,13], while cervical cytobrushes sample the cervical os, collecting cells derived from the epithelium of endocervix and presumably the transformation zone. Cytobrush samples therefore sample a region composed of a single layer of columnar epithelium, while biopsies, include a region lined by a squamous stratified epithelium[5]. As a result, the nature of the leucocyte populations collected by cervical biopsy and cytobrush differs. Biopsies collect a higher proportion of CD3+ T cells, whereas cytobrushes result in collection of a higher proportion of CD14+ monocytes/macrophages[13].

Studying the immunology of the FGT has been an interest for many years[20-22] and we have accumulated a great deal of expertise with the study of cytobrush-derived CMCs. Our studies focus mostly on the study of HIV-infected, uninfected and HIV-exposed seronegative (HESN) female sex workers from Nairobi, Kenya. HIV preferentially replicates in activated T cells[23] and lower numbers of activated cells that can be targeted by HIV in the FGT could contribute to protection against HIV acquisition. In line with this hypothesis, several studies have described lower immune activation among HESN sex workers who are highly exposed to HIV yet remain uninfected[24,25], and this quiescent phenotype is also observed in the FGT[14]. Here, we describe methodology for processing and assessing T cells activation in CMC samples derived from cervical cytobrushes by *ex vivo* flow cytometry.

Protocol

Ethics statement: The research ethics boards of both the University of Manitoba and Kenyatta National Hospital/University of Nairobi approved this study and written informed consent was obtained from all study participants.

1. Preparation of Media and CMC Collection Tubes

1. Prepare phosphate buffered saline (PBS) solution (137.93 mM NaCl, 2.67 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4). Autoclave for sterility. This can be stored at 4 ºC for several months.
2. Pre-aliquot 5 ml PBS into 50 ml falcon tube (one per sample to be collected) and store at 4 ºC until sample collection time.
3. Label 2-4 cryovials or 1.5 ml microcentrifuge tubes per sample to collect lavage for storage.
4. Prepare cell culture media: RPMI 1640 + 1% penicillin/streptomycin/amphotericin B (final concentration 100 units/ml, 100 µg/ml and 250 ng/ml, respectively, with no FCS/FBS added.

2. Collection of Cytobrush Samples

Collection of cytobrush samples is a non-invasive procedure that must be performed by a trained MD or gynecologist.

1. Collect cervical mononuclear cells (CMC) using both a cytobrush and a wooden or plastic scraper. Collect CMC from the participant under speculum examination by inserting the scraper and rotating around the cervical os (**Figure 1**). Insert the cytobrush into the endocervical os, rotate 360°, and immediately place both the cytobrush and scraper into the 50 ml falcon tube containing 5 ml of PBS.
2. Keep samples on ice/at 4 ºC until processing. To maintain cell viability, process samples within 2 hr of collection.
3. If possible, collect matched blood samples in green top heparin vacutainers for peripheral blood mononuclear cell (PBMC) isolation to serve as a comparator/control for CMC analysis.

3. Isolation of CMCs

Perform sample processing in a biosafety level 2 laboratory, in a sterile biosafety cabinet with double gloves.

1. During collection, CMC samples can become contaminated with blood. Exclude samples with visible blood contamination from the study in order to prevent confounding inclusion of PBMC in the final sample. Due to the low number of lymphocytes isolated from CMC samples, minor blood contamination can easily overwhelm the CMC lymphocyte component.
2. Vortex falcon tubes containing both the cytobrush and scraper for 45 sec to dissociate cells from the brushes. Samples may become foamy during this process; this is normal.
3. Use the cytobrush to scrape any remaining material off the scraper, and discard the scraper into a bleach solution. To collect any cells remaining attached to the brush/scraper, use a fresh glove to squeeze the scraper between the thumb and forefinger. Slide down the brush, allowing the cells and PBS to be collected in the same 50 ml tube. Discard the cytobrush into a bleach solution, and discard the glove. IMPORTANT: Use a new glove for each sample.
4. Fit a 100 µm nylon cell strainer to a fresh 50 ml falcon tube. Using a transfer pipette, collect the PBS-cell suspension from the sample tube and filter through the strainer into the fresh tube.
5. Add 5 ml of RPMI 1640 to the original sample tube. Using the transfer pipette, wash the sides of the sample tube with the RPMI, and transfer through the filter to the new collection tube. Wash the bottom of the nylon filter with the RPMI as well.
6. Centrifuge the samples for 10 min at 514 x g. It is crucial to leave the centrifuge brake off during this step, in order to prevent dislodging of the CMC pellet.

7. Gently remove the supernatant from the tube, taking care to not disturb the pellet. Pellet size will be highly variable; in some samples, the pellet is quite large due to the presence of large numbers of epithelial cells, while in other cases, the pellet is barely visible and highly transparent.
8. Resuspend the pellet by gentle agitation of the falcon tube. Add 5 ml of PBS to wash the sample.
9. Centrifuge again for 10 min at 514 x g with no centrifuge brake.
10. Carefully discard the supernatant and re-suspend the pellet as above. The cells are ready for either cryopreservation (using a PBMC cryopreservation protocol), stimulation or flow cytometry phenotyping.

4. CMC Surface Staining and Flow Cytometry

1. Resuspend the pellet from step 3.10 in 100 µl of blocking solution and transfer either into a 96-well plate or FACS tube. The blocking solution (to block Fcγ receptors) recipe is as follows: 1.8 µl mouse IgG (final concentration 0.2 µg/µl), 5 µl FBS and 93.2 µl FACS wash (PBS +2%FBS). Staining can be performed in a 96 well plate if pellet sizes are small enough, but may need to be performed in FACS tubes if pellets are routinely large. It is important to titrate antibodies accordingly.
2. Block the samples for 10 min on ice/at 4 °C.
3. Wash the cells with 100 µl of FACS Wash (PBS+2% FBS) in 96-well plates, or 500 µl in FACS tubes. Centrifuge at 600 x g for 10 min at 4 °C with the brake on low (or off, if a low brake setting is unavailable).
4. Remove the supernatant and re-suspend the cell pellet by agitation. Add viability Stain (Live Dead fixable viability dye). Prepare viability dye according to manufacturer's instructions. It may need to be titrated for use in each lab/cytometer setup. Incubate for 30 min in the dark at 4 °C.
5. Wash the cells 100 µl/500 µl of PBS (for 96-well plate/FACS tubes). Centrifuge at 600 x g for 10 min with low/no brake. Remove supernatant and resuspend cells.
6. Incubate the cells with the cocktail of surface marker antibodies, in a total volume of 100 µl. IMPORTANT: Optimize and titrate each flow cytometry panel prior to sample staining. Incubate cells for 30 min in the dark at 4 °C.
7. Repeat step 4.5. if staining in a 96-well plate, transfer cells to a FACS tube and dilute to a final volume of 750 µl of FACS Wash containing 1% paraformaldehyde (PFA) (or CytoFix, diluted 1 in 4). Proceed to data acquisition on a flow cytometer.

5. Collection and Preparation of Cervical Vaginal Lavage (CVL)

1. Prior to cytobrush sampling, use a syringe to wash the endocervix with 2 ml of sterile PBS and aspirate the lavage from the posterior fornix.
2. Collect the aspirated lavage sample into a 15 ml falcon tube and keep on ice/at 4 ºC until processing.
3. Centrifuge sample at 400 x g for 7 min to remove cellular debris.
4. Collect the supernatant, aliquot the sample into 1 ml or 500 µl aliquots, and store at -70 °C. Aliquots can be thawed for ELISA or bead array analysis of CVL proteins, but avoid multiple freeze/thaw cycles.
5. If desired, resuspend the pellet of cellular debris in RNA Later to measure RNA expression by following the manufacturer's protocol.

6. Data Acquisition

1. Prepare a set of compensation tubes that matches the antibody panel. Run the compensation tubes to adjust spectral overlap. Run the samples on any multicolor flow cytometer that is configured for the fluorochrome conjugated antibodies used in the panel.
2. Acquire a PBMC sample first to adjust Forward Scatter (FSC) and Side Scatter (SSC) voltage in order to detect the lymphocyte population. Try to keep them at 100 on each axis on a linear scale. Running a PBMC control will make it significantly easier to find the CMC lymphocyte population.
3. The FSC threshold for CMC samples may need to be increased relative to PBMC samples due to smear up the SSC axis at low FSC values.
4. Vortex each tube before acquiring. Acquire the entire tube to maximize the number of lymphocyte gate events collected. Monitor the machine carefully to avoid clogging.
5. Export the data into a flow cytometric analysis program such as FlowJo.

7. Gating Strategy

1. Select Forward side scatter high (FSC-H)/Forward side scatter area (FSC-A) to determine the singlet population.
2. Select Time versus Fluorescent marker (as example FITC) to control for the quality of the acquisition. Change in flow rate may introduce artifacts in the data. Exclude any area that shows discrepancy in flow rate.
3. Identify the lymphocyte population on SSC-A/FSC-A plot. Use a PBMC control to facilitate the identification. Note that the CMC lymphocyte population might not be as clear as the PBMC control.
4. Gate on SSC-A/viability dye to exclude dead cells from live cells. Dead cells can non-specifically incorporate the antibodies, which will introduce artifacts.
5. Gate on the SSC-A/CD3 to identify the T cell population. From this gate, identify the CD4+ and CD8+ populations.

Representative Results

Multiparameter flow cytometry is a powerful tool to dissect the phenotypes and functions of cell subsets in previously uncharacterized tissues. Analysis of CMC samples can yield information on both lymphocyte and monocyte populations with appropriate gating strategies.

A representative CMC gating strategy, compared to a matched PBMC profile, is shown in **Figure 2**. The FSC-A versus FSC-H plot allows for the exclusion of cell doublets, which are highly prevalent in CMC samples compared to PBMC, even after filtration (**Figure 2A**). Quality control includes the removal of flow issues by gating on time, where changes in flow rate or artefacts due to sample clumps can be easily identified

(**Figure 2B**). Identification of the lymphocyte/monocyte populations is relatively similar in both PBMC and CMC samples (**Figure 2B**). Exclusion of dead cells by viability dye prevents inclusion of apoptotic cells that have non-specifically taken up fluorescent antibody conjugates. Viability is lower in CMC samples compared to PBMC, and can be highly variable from sample to sample (**Figure 2B**).

This analysis will focus on phenotyping the T lymphocyte population, but CMC samples also contain monocytes. After gating on the monocyte population, cells are gated based on a viability dye and a dump channel (**Figure 3A**). The dump channel contains antibodies against CD56, CD3 and CD19, and gating on dump channel-negative cells will eliminate any lymphocyte contamination in the monocyte population. Cells can then be identified based on the expression of markers such as CD16 and CD11c. The CD3+ lymphocyte population is typically reduced in proportion among CMC compared to PBMC (**Figure 3B, Table 1**). The high proportion of epithelial cells, granulocytes and non CD3+CD45+ leucocytes contained in CMC samples dominates over the T cell population. CD4+ T cells (median: 55.30%, IQR: 50.53%-68.18%) and CD8+ T cells (median: 25.60%, IQR: 21.50%-33.80%) are found at a lower ratio (closer to 2:1 among CMC) compared to the 5:1 ratio observed among healthy PBMC samples from the commercial sex worker cohort in Kenya (**Figure 3C, Table 1**). As in blood, HIV infection affects the proportions of CD4+ and CD8+ T cells among CMC, decreasing the CD4+:CD8+ ratio to 0.7 (median: 0.7, IQR: 0.31-2.45) (**Figure 3C,Table 1**). However, it is not uncommon to observe a higher proportion of CD8+ T cells among the CMC populations from healthy individuals (**Figure 3C**). Interestingly, an expanded CD4-CD8- (double negative, DN) T cell population relative to PBMC DN T cell frequency can be observed among many CMC samples of HIV-negative individuals (**Table 1**). These results are similar to those obtained for foreskin samples[26], although these cells are poorly characterized.

Typical T cell phenotypic markers are easily quantified among CMCs but may differ from PBMCs; **Figure 4** demonstrates the identification of a CD8+CD161++ (MAIT) population that is distinct in PBMCs but almost absent in CMCs (**Figure 4A**). Expression of activation markers CD69 and HLA DR, migration marker CCR5 or exhaustion marker PD-1 can be clearly identified on CD4+ or CD8+ populations (**Figure 4B**).

Quantification of the concentration of cytokines/chemokines in CVL can be analyzed in parallel with CMC staining. This allows for correlation between the CVL cytokine milieu (pro-inflammatory versus immunoregulatory) and the phenotype of CMCs. These data can also be used to determine whether the frequency of chemokine receptor expression on CMCs is related to the relative expression of either plasma or CVL chemokines[14]. **Table 2** indicates the mean concentration of cytokine and chemokine analytes in CVL collected from healthy women and assayed by Milliplex cytokine bead array kits.

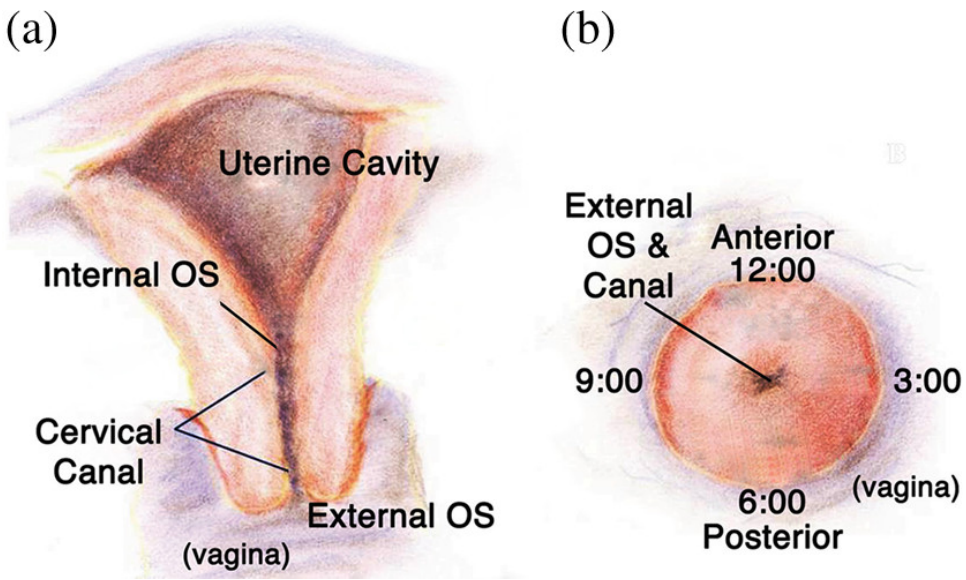

Figure 1. Anatomy of the female reproductive tract. A) Cross-sectional view of the female reproductive tract, showing the relationship of the vagina to the external cervical os, cervical canal and uterine cavity. **B)** Front angle view of the cervix, showing the external os, and the anterior and posterior fornix regions at 12:00 and 6:00, respectively. Reproduced with permission from Reusch *et al*[27]. The cervical scraper is rotated around the cervical os to collect CMC, while the cytobrush is inserted into the cervical canal and rotated to collect additional CMC. Cervical vaginal lavages (CVL) are collected by washing the endocervix with PBS and collecting it from the posterior fornix.

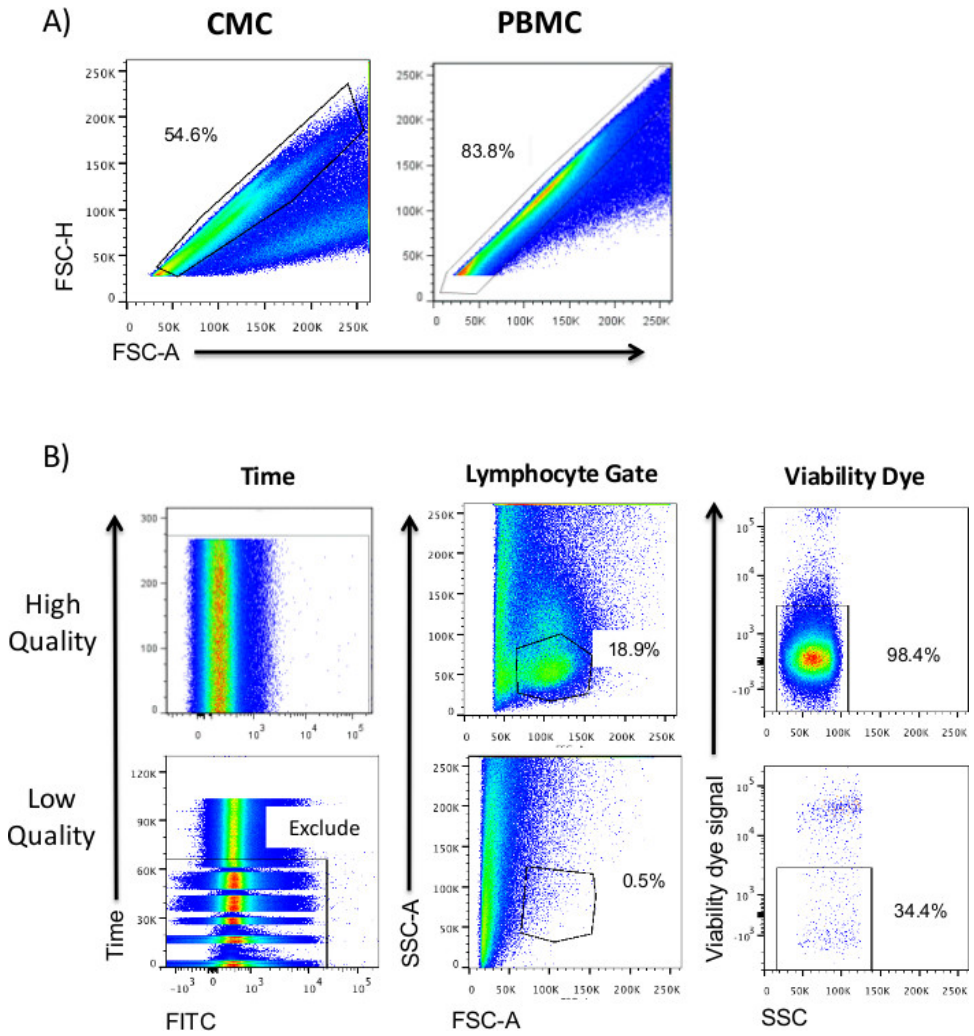

Figure 2. Gating and quality control of CMC samples. A) Identification of singlets by FSC-A versus FSC-H plot demonstrates the low percentage of singlets in CMC samples compared to PBMC samples, even after filter-based CMC isolation. **B)** Examples of high and poor quality samples are shown for several quality control steps. Gating of Time versus fluorescence (or SSC/FSC) can identify flow rate issues and be used to exclude events that may result in staining artifacts. A lymphocyte population based on FSC versus SSC may or may not be easily identified depending on the sample. Inclusion of a viability dye is crucial, as some samples may contain fewer than 50% viable cells. Viability (amine-reactive) dyes stain dead cells more brightly than live cells, as loss of membrane integrity upon cell death allows the dye to access amine groups on proteins within the cell. Viable cells are therefore identified by gating on the viability dye-negative population.

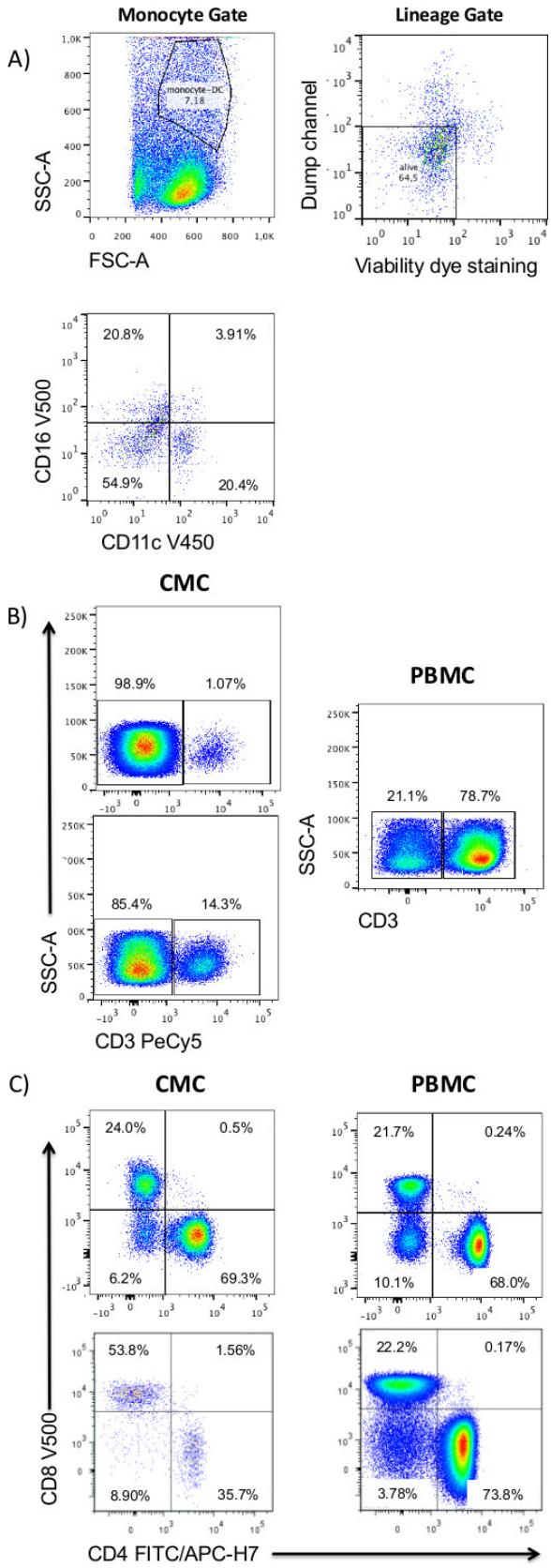

Figure 3. Identification of T cell subsets. A) The CD3+ population among CMC samples is generally a smaller proportion of the lymphocyte gate compared to PBMC samples. CMC samples are also more variable in the size of the T cell population, as shown. **B)** CD4+, CD8+ and CD4-CD8- T cell populations are easily identified in both CMC and PBMC samples. CD4:CD8 T cell ratio can vary from sample to sample among CMCs, as shown. Data shown was collected from HIV-uninfected women.

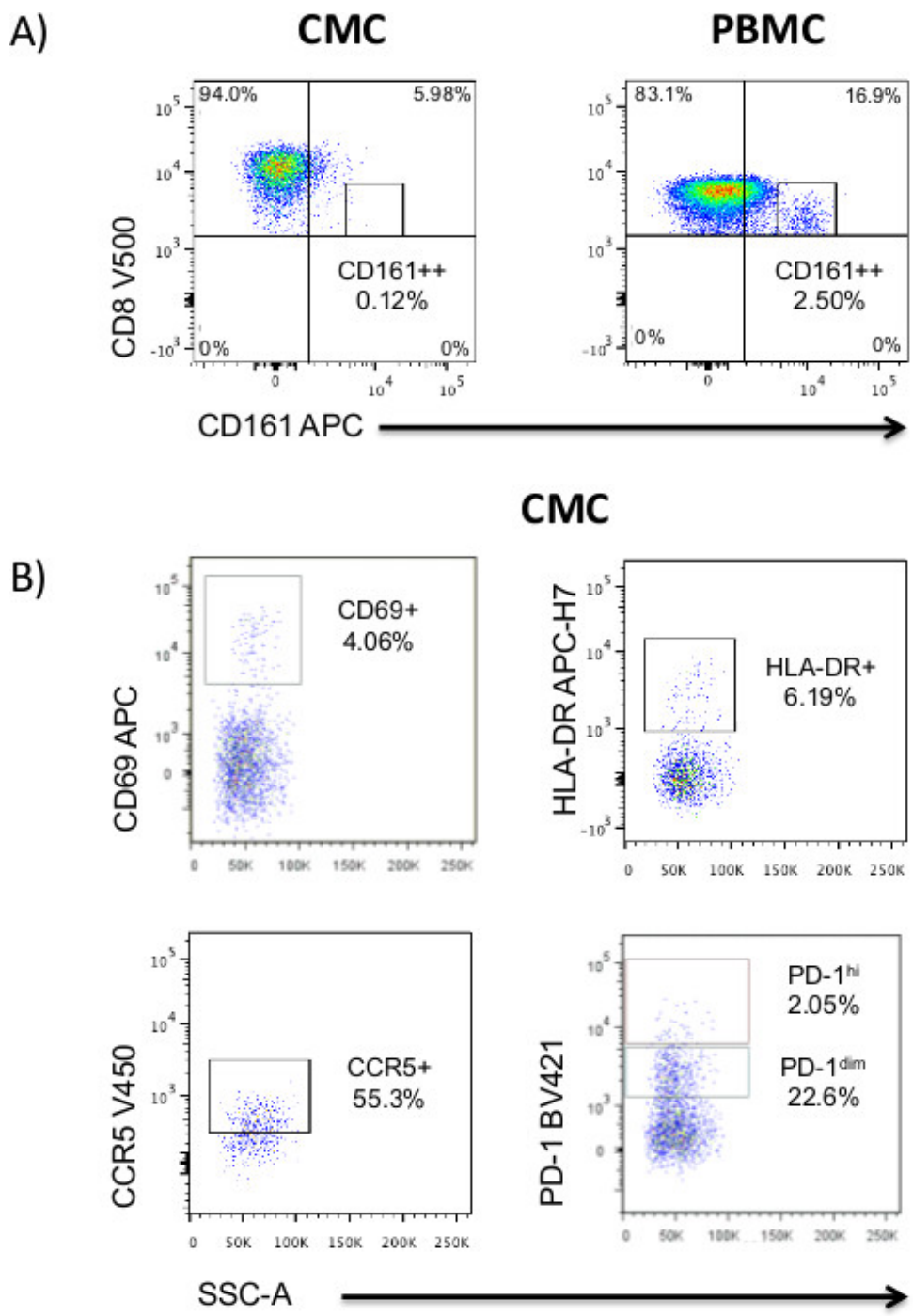

Figure 4. T cell phenotypes in CMC samples. A) The mucosal associated invariant T cells (MAIT) CD8+CD161++ population that is easily identified in PBMC samples is almost entirely absent in CMCs. The CD8+CD161+ T cells may be identified in both samples. **B)** CMCs exhibit expression of phenotypic markers including CD69, HLA DR, CCR5 and PD-1. Gates were drawn based on fluorescence minus one (FMO) controls. Data shown was collected from HIV-uninfected women.

	CMCs			PBMCs		
Population	HIV+ (n=34)	HIV- (n=44)	P value[a]	HIV+ (n=28)	HIV- (n=35)	P value[a]
	median (IQR)	median (IQR)		median (IQR)	median (IQR)	
%Live lymphocytes	85.65 (60.25-92.63)	88.70 (74.85-95.38)	0.1193	-	-	
%CD3+ in live lymphocyte gate	5.94 (0.90-16.80)	1.44 (0.39-8.46)	0.0303	41.15 (17.08-62.10)	41.90 (20.40-51.30)	0.4231

p value[b]	< 0.0001	< 0.0001				
%CD8+ in CD3+ gate	43.20 (25.75-66.00)	25.60 (21.50-33.80)	0.001	30.05 (22.75-38.78)	14.60 (8.01-24.80)	< 0.0001
p value[b]	0.0291	< 0.0001				
%CD4+ in CD3+ gate	31.10 (20.45-63.10)	55.30 (50.53-68.18)	0.0003	56.10 (50.10-61.28)	77.20 (65.90-92.80)	< 0.0001
p value[b]	0.002	< 0.0001				
Ratio CD4:CD8	0.71 (0.31-2.45)	2.09 (1.55-3.01)	0.0003	1.92 (1.44-2.39)	5.34 (2.66-10.77)	< 0.0001
p value[b]	0.004	< 0.0001				
%DN in CD3+ gate	10.40 (6.36-14.30)	10.70 (6.76-19.10)	0.4793	9.58 (6.65-15.98)	7.01 (5.26-8.07)	0.0032
p value[b]	0.8338	0.0006				

Table 1. Relative proportions of T cell subsets in CMCs and PBMCs. CMCsamples from 44 HIV-negative and 34 HIV-positive female sex workers and PBMC samples from 35 HIV-negative and 28 HIV-positives were compared for their relative proportion of live cells, CD3+ T cells and CD4+, CD8+ and CD8-CD4- DN T cell subsets. Data are presented as median (25^{th}-75^{th} interquartile, IQR). Differences between groups were calculated by Mann-Whitney test. p values indicate the outcome of a comparison between [a]HIV+ and HIV- or [b]CMC and PBMC data.

Analyte	Mean[a] (Std dev.)	Detection limit
	pg/ml	pg/ml
MIP-3a	35.5 (90.6)	2.9
MIG	1447 (3026)	19.4
ITAC	2.7 (6.3)	0.8
Fractalkine	51.6 (69.5)	10.6
IFN-a2	24.0 (12.0)	40.6
IFN-g	3.9 (14.2)	0.3
IL-1a	268.4 (721.3)	6.4
IL-1b	46.8 (126.2)	0.7
IL-1ra	4967.0 (3597)	5.5
IL-2	0.9 (2.6)	0.6
IL-6	14.8 (30.5)	0.7
IL-7	6.3 (10.1)	0.1
IL-8	1491 (2126)	0.3
IL-10	4.5 (8.6)	0.5
IL-15	1.4 (2.7)	0.7
IL-17	1.1 (2.2)	0.3
IP-10	381.4 (1100)	2.2
MCP-1	129.4 (340.4)	1.6
MCP-3	5.9 (7.1)	3.7
MDC	84.2 (94.6)	6.9
MIP-1a	20.9 (28.9)	6.4
MIP-1b	28.5 (42.6)	8.9
sCD40L	10.8 (19.3)	9
sIL-2Ra	8.4 (9.8)	7.7
TNF-a	1.9 (4.1)	0.1

Table 2. Expression of cytokines and chemokines in CVL. Samples from 51 HIV-uninfected (non-HESN) participants of the female sex worker cohort at mid-menstrual cycle were assayed for cytokine/chemokine concentration using the Milliplex MAP Human Cytokine/Chemokine bead array kit according to the manufacturer's overnight protocol, assayed in duplicate. IFN: interferon; MCP: monocyte chemotactic protein; MDC: macrophage derived chemokines, s: soluble; TNF: tumor necrosis factor; MIP: macrophage inflammatory protein; ITAC: interferon

inductible T cell alpha chemoattractant; MIG: monokine induced by gamma interferon; IP-10; interferon inductible protein. [a]Values below the limit of detection were assigned a value of half the limit of detection.

Discussion

Given the large gaps in knowledge with respect to immunity at the female genital tract (FGT), phenotypic analysis of CMCs can provide a wide array of insights into multiple lymphocyte populations at the cervix. Coupled with proteomic analysis and viral load measurements in cervical lavage, immunity to sexually transmitted infections (STI)s and other pathogens can be dissected in various populations.

Technical considerations - CMCs: The isolation and successful staining of CMC samples can be challenging. Optimization of the CMC collection protocol has emphasized the effectiveness of collecting the CVL first, followed by the cervical scraper and then finally the cytobrush. It is important to increase planned sample sizes compared to those calculated for peripheral blood studies due to the increased likelihood of sample exclusion for a variety of reasons, discussed below.

Testing of the desired flow panel on CMC samples is critical prior to study initiation. It is important to monitor cell autofluorescence in CMC samples, particularly in the monocyte gate. CMC autofluorescence in some channels can be elevated compared to PBMCs, which may affect gating and signal-to-noise ratios. Furthermore, optimum voltages should be confirmed for CMC samples if previously determined on PBMC samples.

To preserve data integrity, it is important to exclude samples with blood contamination or confounding STIs. The inclusion of a cell viability dye in the flow cytometry panel is vital to exclude dead cells that non-specifically take up fluorescent antibodies. While most samples are highly viable, it is not uncommon to exclude 10-20% of cells by viability dye. If subset analysis based on CD4 and CD8 expression is planned, it is important to obtain at least 100 CD3+ events to proceed with analysis. Samples collected from different women, and even from the same woman at different time points, can yield vastly different cell numbers[13].

Flow cytometric analysis of mucosal lymphocytes is often easier and more successful if a PBMC control sample can be run simultaneously with the CMC samples. In some CMC samples, the lymphocyte population is difficult to identify by FSC/SSC gating, but inclusion of the PBMC sample will give a better idea of where to gate. Even in samples without a clear lymphocyte FSC/SSC population, distinct CD3+ populations can be identified. If the data acquisition is interrupted by technical issues (cytometer intake clogging, bubbles, etc.) gating on fluorescence over time can remove data collection artifacts while still allowing sample analysis. Appropriate controls such as fluorescence minus one (FMO) tubes for gate placement may need to be performed on peripheral blood mononuclear cells (PBMC) when the number of cells required is too substantial to use cytobrush samples.

Multiple studies have demonstrated the detection of antigen-specific T cell responses among CMC samples. CMC cytokine production can also be induced by PMA/Ionomycine, PHA, IFNγ and anti-CD3 stimulation. One of the major limitations of CMC stimulations is cell number, which reduces the number of conditions and controls that can be performed per sample. It is also crucial to carefully monitor and take extra steps to prevent cell culture contamination. The genital tract is not a sterile site, and in some cases extra antibiotics must be added to culture media to prevent bacterial overgrowth. Although CMCs can be cryopreserved with little loss of viability, recovery is approximately 50%, making cryopreserved samples poor candidates for stimulation studies unless cell expansion is planned[10].

Technical considerations – CVLs: Collection of CVL usually results in limited sample volume, preventing the analysis of a large number of protein concentrations by repeated ELISA. An alternative assay is the cytokine bead array, based on the Luminex platform or Flow Cytometry platform. Multiplexed kits are available from several commercial sources, allowing for the quantification of up to 30 analytes in an extremely small sample volume (often ~25 µl). Optimization of these assays has revealed optimal detection of analytes using the overnight incubation protocol. Some analytes that are detectible in plasma/serum samples are not detectible in CVL. Notably, concentration of CVL samples using spin columns did not improve detection of any analytes, suggesting that there is no benefit to sample concentration prior to running the bead array assay.

Confounders specific to the genital mucosa: Studies of FGT immunology must take care to acknowledge and account for as many confounding variables as possible. The menstrual cycle has now been demonstrated to impact peripheral blood immunity[28], and likely exerts greater impacts on CMC phenotype and cell composition as well as the proteins collected from CVL samples[29]. Synchronizing sample collection to menstrual cycle phase is the preferred way to reduce data variability, whether measured by estrogen/progesterone levels or days since the first day of last menses. The use of hormonal contraception also greatly influences the mucosal environment and its effect should be taken into consideration when designing or analyzing a study. Alteration of the vaginal flora resulting in bacterial vaginosis (BV) also modifies the immune environment and cellular phenotypes. BV can be diagnosed by Gram staining and a Nugent score can be established to control for the confounding effect of BV.

Sexual activity also has a profound impact on genital immunology, since allo-responses to semen can induce rapid and dramatic changes in cell phenotype and trafficking. Indeed, Lajoie *et al*. have described differences in CVL protein composition between sex worker and non-sex worker populations, with changes occurring in the first few years after initiation of sex work.

Finally, cultural practices such as douching will introduce further variation into mucosal sampling. Depending on the reagent used (bleach, detergent, lime juice, etc.), douching may reduce the frequency/phenotype of cell populations, or alter autofluorescence compared to other samples. Ideally, douching prior to cytobrush sampling should be discouraged among donors.

Future applications: In addition to yielding important insight into regulation of mucosal immunity during menstrual/hormonal variability, viral/bacterial infection and before/after menopause, analysis of mucosal samples is particularly relevant to vaccine studies against STIs/mucosal pathogens. In the case of HIV, where a major goal of vaccine studies is to elicit a mucosal antibody or T cell response, development of CMC-based assays will play a crucial role in determining correlates of protection.

Disclosures

The authors have nothing to disclose.

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