Video Article

Isolation and Flow Cytometric Analysis of Human Endocervical Gamma Delta T Cells

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Abstract

The female reproductive tract (FRT) mucosal immune system serves as the first line of defense. Better knowledge of the genital mucosa is therefore essential for understanding pathogenicity of different pathogens including HIV. Gamma delta (GD) T cells are the prototype of 'unconventional' T cells and represent a relatively small subset of T cells defined by their expression of heterodimeric T-cell receptors (TCRs) composed of gamma and delta chains. This sets them apart from the classical and much better known CD4⁺ helper T cells and CD8⁺ cytotoxic T cells that are defined by alpha-beta TCRs. GD T cells often show tissue-specific localization and are enriched in epithelium. GD T cells orchestrate immune responses in inflammation, tumor surveillance, infectious disease, and autoimmunity.

Here, we present a method to reproducibly isolate and analyze human endocervical intraepithelial GD T lymphocytes. We have used endocervical cytobrush samples from women participating in the Women's Interagency HIV Infection Study (WIHS). Knowledge about GD T cells interactions during conditions in which there is an insult to the vaginal mucosal could be applied to any clinical study in which mucosal vulnerability is addressed, including the development of vaginal microbicides. In addition, knowledge about mucosal GD T cell responses has potential for application of GD T cell-based immune therapy in treating infectious diseases.

Video Link

The video component of this article can be found at <https://www.jove.com/video/55038/>

Introduction

We developed methodology of using endocervical brush samples to evaluate intraepithelial GD T cells. The endocervical canal is lined by a single layer of columnar epithelium. Intraepithelial lymphocytes represent frontline lymphocytes residing within the epithelial layer that can rapidly initiate immune responses upon encountering pathogenic microbes^{7,8}. Understanding the immunological events of endocervical intraepithelial compartment is important for the design of effective strategies to prevent infections, including HIV.

Our method can be applied for freshly collected human endocervical samples as well as frozen endocervical cells to further explore the role of intraepithelial GD T cells in women with HIV infection or at risk for HIV infection. The main goal of this protocol is to discover novel immunological events in endocervical intraepithelial compartment and to evaluate GD T cell responses as a marker of mucosal vulnerability in women with HIV infection.

The substantial gaps in knowledge around FGT immunity are partially due to the difficulty in successfully collecting and processing mucosal samples. Furthermore, the complex heterogeneity of mucosal immune cells, and their interconnectedness with each other, are major challenges to identifying clinically relevant measurements that reflect the state and capability of the immune system. We developed a method to obtain highly purified intraepithelial GD T cells that can be further analyzed using highly multiplexed, single-cell technologies that may be critical for identifying the underlying mechanisms of FRT immunity.

Protocol

Study activities took place at University of Miami HIV Research Unit in collaboration with the Miami Women's HIV Interagency Study (WIHS) and the Miami Center for AIDS Research (CFAR). Institutional Review Board (University of Miami Miller School of Medicine) approval was obtained prior to recruitment and any assessment or study related procedures.

1. Endocervical Brush Sample Collection

NOTE: Participants underwent a vaginal examination performed by trained MD or gynecologist and collection of intraepithelial lymphocytes were performed under speculum examination by inserting cytobrush.

1. Participant characteristics

1. Recruit women participants who are aged 18 to 45 years of age, sexually active, not pregnant and not on contraceptive medications or with an intrauterine device.
2. Before vaginal examination, have participants undergo a 10 mL blood draw in green top heparin vacutainers for peripheral blood mononuclear cell (PBMC) isolation. to serve as a control for endocervical intraepithelial analysis⁹.

2. Collection of the endocervical samples

1. Insert a sterile speculum of appropriate size into the vagina without lubrication. Use a warm water to facilitate insertion of the speculum and sterile gauze to clean the mucus. The position of the speculum allows for complete visualization of the os and ectocervix.
2. Insert endocervical brush into the endocervical canal until only the bristles closest to the hand are visible. Rotate the brush clockwise for 360°. Transfer the brush into a 15 mL tube containing 5 ml of IMDM medium.
3. Immediately after collection, place the tube containing cytobrush on ice. To obtain high viability of collected intraepithelial cells, deliver the cytobrush to the lab and process within 1 h.

2. Endocervical Cytobrush Sample Processing

1. Vortex tube containing endocervical cytobrush applying 4 short (10 sec) strokes.
2. Centrifuge the tube, containing cytobrush, for 10 min at 250 x g.
3. Carefully remove the cytobrush from the tube (do not disturb the pellet on the bottom of the tube) and add 1 mL of IMDM media and place the tube on ice.
4. Place the cytobrush on the 100 mm cell strainer on top of the 50 ml tube.
5. Wash the cytobrush with 20 ml IMDM.
6. Centrifuge tube for 10 min at 250 x g.
7. Decant the supernatant and pelleted cells resuspend in 1 mL IMDM and combine with the already re-suspended pellet described in 2.3.
8. Count the cells using an automated cell counter¹⁰ or count the cells using hemocytometer by combining 10 µL of the cell suspension with 10 µL of trypan blue dye. Gently pipet up and down ten times to mix the cells and dye. Load 10 µL of the mixture into the opening of either of the two hemocytometer chambers. Count all the cells in the five grid areas. Keep a separate count of the viable and non-viable cells. Determine the cell viability using the following formula: % Viable cells = Non-viable cells x 100/total cell number cells.
Determine the subsequent cell concentration per ml (and the total number of cells) using the following calculations. Total Cells per ml = total cells counted x (dilution factor/ number of counted square) x 10,000 cells/ml.
9. Freeze isolated endocervical cells using freezing medium containing 10% DMSO and 90% FBS.

3. Flow Cytometry

1. Re-suspend 1-3 x 10⁵ endocervical cells in 100 µL FACS buffer (PBS containing 1% bovine serum albumin
2. Add 1 µL/10⁶ LIVE/DEAD Fixable Yellow Dead Cell Stain kit and antibodies for surface markers at the concentrations described in **Table 1**.
3. Incubate the cells for 30 min at +4 °C, protected from light.
4. Add 1 mL FACS buffer and the cells and centrifuge tubes for 5 min at 350 x g
5. Re-suspend cell pellet in 300 µL 1% paraformaldehyde.
6. Prepare compensation controls using beads and antibodies used for staining by adding 100 µL of FACS buffer and one drop of the beads and the same concentration of the antibodies used for sample staining.
7. Acquire stained beads and samples on a flow cytometer, equipped with 405 nm, 488 nm, and 635 nm lasers.

4. Endocervical Gamma delta T Cell Isolation

1. Magnetic bead isolation

1. Re-suspend endocervical cell pellet ($\leq 10^7$ total cells), described in 2.6, in 40 µL of buffer included in the TCR gamma delta microbead kit referenced in the Table of Materials and Reagents.
2. Follow the kit instructions for two-step staining protocol: first, with anti-TCR gamma delta Hapten-Antibody and second, with anti-hapten microbeads-FITC
3. After incubation of 15 min at 4-8 °C, wash the cells by adding 1 mL of buffer and centrifuge at 300 x g for 10 min. Decant the supernatant by inverting the tube, and tap dry with the help of a piece of paper.
4. Re-suspend the cell pellet in 500 µL of provided buffer and prepare the magnetic column by placing it in appropriate magnetic separator according to the vendor's protocol.
5. Apply cell suspension onto the column and collect unlabeled cells which pass through and wash column 3x with appropriate amount of buffer (500 µL). Perform washing steps by adding buffer three times, each time once the column reservoir is empty. Collect total effluent. This is the unlabeled, negative cell fraction
6. Remove the column from the magnetic separator and pipette 1 ml of buffer onto the column and immediately flush out fraction with the magnetically labeled cells by firmly applying the plunger supplied with the column.
NOTE: Isolated gamma delta T cell are already fluorescently stained for flow-cytometric analysis since magnetically labeled Anti-Hapten microbeads are conjugated with fluorescent dye FITC.

2. Cell sorting

1. For cell sorting, label endocervical cells with viability dye and antibody panel as described in section 3.
2. Set up electronic gates on the live, CD45⁺ CD3⁺ and gamma delta positive cells and perform cell sorting.

Representative Results

Recently, we analyzed endocervical intraepithelial GD T cells and we were the first one to report about the loss of endocervical gamma delta T cells in HIV infected women^{9,11}. Here, we describe the protocol to isolate and analyze the subset of endocervical gamma delta T cells. As shown in **Figure 1**, GD T cells can be readily detected in the human endocervical cell samples.

A unique population of endocervical gamma delta V1 (GD1) T cells was described using multiparameter flow cytometry-based immunophenotyping (**Figure 1**). A representative gating strategy for HIV uninfected endocervical sample is shown in **Figure 1**. Analysis was performed on the total endocervical cells by setting the electronic gate on Forward/Side scatter (**Figure 1A**) followed by the exclusion of cell doublets (**Figure 1B**). Viable cells were defined by exclusion of dead cells using LIVE/DEAD Fixable Yellow Amine Dead Cell Stain (**Figure 1C**). Analysis of CD45 expression was performed on live gated cells (**Figure 1D**), followed by the analysis of CD3 expression (**Figure 1E**). Gamma delta 1 (GD1) or gamma delta 2 (GD2) positive cells were defined as a subpopulation of CD3⁺ cells that express GD TCR (TCR gamma delta V1 or TCR gamma delta V2). Furthermore, it was confirmed that only CD3⁺ T cells express TCR gamma delta since gated CD3⁺ T cells do not express TCR gamma delta V1 or delta V2. Phenotypic analysis of gated CD3⁺GD1⁺ cells revealed (**Figure 1G**), that majority of GD1 cells (~74%) are CD4⁺CD8⁻.

In the present study, feasibility of utilizing endocervical brush samples to purified intraepithelial GD T cells by positive magnetic selection was demonstrated (**Figure 2**).

Figure 1: Gamma delta (GD) T cell subpopulation in endocervical mucosa analyzed by flow cytometry. Following gating strategy was used to identified endocervical T cell populations: (A) endocervical cytobrush cells were gated according the size and granularity (FSC, forward side scatter vs SSC, side scatter). (B) Cell doublets were excluded by setting the electronic gates in the FSC-A (area) vs SSC-W (width). (C) Live cells are gated on the viability dye-negative population. (D) Electronic gate was set on live, CD45 positive cells. (E) Electronic gate for CD45 positive cells was set on CD3 positive cells (F) TCRVD1 (GD1) and TCRVD2 (GD2) expression was analyzed on CD3⁺ cells. (G) CD4 and CD8 expression was analyzed on GD1⁺ cells. (H) TCRVD1 and TCRVD2 expression was analyzed on gated CD3 negative cells. [Please click here to view a larger version of this figure.](#)

Figure 2: Isolation of endocervical gamma delta T cells (GD) by positive magnetic separation. Frequency of endocervical GD T cells was confirmed before and after positive magnetic separation. Endocervical cells were first labeled with anti-TCR gamma delta antibody and then with fluorescently stained with Anti-Hapten microbeads-FITC. Percent of TCR GD+ T cells was reported before and after magnetic separation. [Please click here to view a larger version of this figure.](#)

Discussion

Assessment of female lower genital tract mucosal vulnerability is an essential component of clinical studies addressing risk of HIV acquisition and transmission. Typically FGT vulnerability to HIV infection is evaluated by measuring soluble immune modulators in genital secretions (cytokines, chemokines and antimicrobial peptides)^{12,13}. However, these biomarkers are only indicative of vaginal inflammation, are affected by physiological and pathological events, and do not always represent direct mucosal damage. In order to improve our understanding of how mucosal factors increase the risk of acquiring and transmitting HIV, cellular markers of mucosal damage need to be developed. GD intraepithelial T cells are present in the FGT, intercalate between epithelial cells, and are thought to contribute to homeostasis as well as to a first line of defense against environmental challenges. GD T cells have the potential to constitute an additional biomarker to assess mucosal vulnerability in the FGT.

In this protocol, it is shown how to prepare intraepithelial lymphocytes from human endocervical cytobrush sample and is easily mastered. The most critical point of this process is to maintain cell viability by fast processing of the sample as well as by keeping the cells on ice during the entire procedure. The cell yield will depend on cytobrush collection technique, handling skill as well as the infectious status (HIV infection or other viral infections, bacterial or fungal infections). Our group and others have published various studies describing different lymphocyte populations in human genital tract^{9,14,15}. In our hands, one endocervical cytobrush sample can provide $0.5-5 \times 10^6$ cells with 80-90% viability. In comparison with the commonly used isolation method^{14,15}, by our method the viability and cell yield is increased.

This method provides a powerful and an efficient tool to study endocervical intraepithelial gamma delta T lymphocytes and could provide insight into other immune cells that migrate to this compartment during infection and/or inflammation. The *in vivo* cellular composition of mucosal tissues is often difficult to investigate in a comprehensive and quantitative way. Techniques such as immunohistochemistry and flow cytometry are limited by the availability of antigen specific monoclonal antibodies and by the small number of parallel measurements that can be performed on each individual cell. Traditional high-throughput assays, such as gene-expression arrays, when performed on whole tissues, provide information on average gene expression level, and can be only indirectly correlated to quantitative modifications in cellular subpopulations. These limitations become particularly difficult to overcome when studying minority populations, or the cell population that lack exclusive markers. By combining "fluorescence activated cell sorting" (FACS) and "single-cell PCR gene-expression analysis" one can perform a high-throughput transcriptional analysis of the intraepithelial GD T cells in endocervix. This method exploits the capacity of modern flow cytometers to sort individual single cells with accuracy and precision, together with the use of microfluidic technologies to perform high sensitivity multiplexed PCR from minute amounts of mRNA, thereby allowing parallel analysis of the expression of up to 96 genes for each individual cell¹⁶.

Disclosures

Authors do not have commercial or other association that might pose a conflict of interest (e.g., pharmaceutical stock ownership, consultancy, advisory board membership, relevant patents, or research funding).

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References

1. Kaul, R. *et al.* The genital tract immune milieu: an important determinant of HIV susceptibility and secondary transmission. *J Reprod Immunol.* **77**, 32-40 (2008).
2. Haase, A. T. Targeting early infection to prevent HIV-1 mucosal transmission. *Nature.* **464**, 217-223 (2010).
3. Hayday, A. C. [gamma][delta] cells: a right time and a right place for a conserved third way of protection. *Ann Rev Immunol.* **18**, 975-1026 (2000).
4. Nakasone, C. *et al.* Accumulation of gamma/delta T cells in the lungs and their roles in neutrophil-mediated host defense against pneumococcal infection. *Microbes and infection / Institut Pasteur.* **9**, 251-258 (2007).
5. Autran, B. *et al.* T cell receptor gamma/delta+ lymphocyte subsets during HIV infection. *Clin Exp Immunol.* **75**, 206-210 (1989).
6. Pauza, C. D., Poonia, B., Li, H., Cairo, C., & Chaudhry, S. gammadelta T Cells in HIV Disease: Past, Present, and Future. *Front Immunol.* **5**, 687 (2014).
7. Hayday, A. C., & Spencer, J. Barrier immunity. *Sem Immunol.* **21**, 99-100 (2009).
8. Cheroutre, H., Lambolez, F., & Mucida, D. The light and dark sides of intestinal intraepithelial lymphocytes. *Nature Rev Immunol.* **11**, 445-456 (2011).
9. Strbo, N. *et al.* Loss of Intra-Epithelial Endocervical Gamma Delta (GD) 1 T Cells in HIV-Infected Women. *AJRI.* **75**, 134-145 (2016).
10. Szabo, S. E., Monroe, S. L., Fiorino, S., Bitzan, J., & Loper, K. Evaluation of an automated instrument for viability and concentration measurements of cryopreserved hematopoietic cells. *Lab Hematol.* **10**, 109-111 (2004).
11. Alcaide, M. L. *et al.* Bacterial Vaginosis Is Associated with Loss of Gamma Delta T Cells in the Female Reproductive Tract in Women in the Miami Women Interagency HIV Study (WIHS): A Cross Sectional Study. *PloS one.* **11**, e0153045 (2016).
12. Masson, L. *et al.* Defining genital tract cytokine signatures of sexually transmitted infections and bacterial vaginosis in women at high risk of HIV infection: a cross-sectional study. *Sex Transm Dis.* **90**, 580-587 (2014).
13. Fichorova, R. N. *et al.* Interleukin (IL)-1, IL-6, and IL-8 predict mucosal toxicity of vaginal microbicide contraceptives. *Biol Reprod.* **71**, 761-769 (2004).
14. Juno, J. A., Boily-Larouche, G., Lajoie, J., & Fowke, K. R. Collection, isolation, and flow cytometric analysis of human endocervical samples. *JoVE.* (2014).
15. McKinnon, L. R. *et al.* Optimizing viable leukocyte sampling from the female genital tract for clinical trials: an international multi-site study. *PloS one.* **9**, e85675 (2014).
16. Dominguez, M. H. *et al.* Highly multiplexed quantitation of gene expression on single cells. *J Immunol Methods.* **391**, 133-145 (2013).