Chapter 23

Isolating Mucosal Lymphocytes from Biopsy Tissue for Cellular Immunology Assays

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Abstract

Mucosal tissues of the gastrointestinal and genitourinary tracts serve as major portals of HIV-1 transmission, and recent literature has highlighted the important role of these tissues in pathogenesis. However, our understanding of human mucosal T-cell responses remains limited. We have previously reported methods for isolating, culturing and analyzing mucosal T-lymphocytes obtained from gastrointestinal biopsy tissue. This method of acquiring tissue is minimally invasive and well accepted by patients, and allows sampling of sites that would not otherwise be accessible without surgical intervention. This chapter summarizes the approach currently in use in our laboratory to isolate and study CD4+ and CD8+ T-cells from rectal biopsies obtained through flexible sigmoidoscopy. These methods are also applicable, with minor modifications, to small tissue samples obtained from other lymphoid tissues.

Key words: Mucosa, lymphocyte, CTL (cytotoxic T-cell).

1. Introduction

The gastrointestinal tract is considered to be the largest lymphoid organ in the body, and contains the majority of the body's lymphocytes. The intestinal mucosa plays an important role in immune defense, and must strike a balance between tolerance to food antigens and commensal microbes, and responsiveness to mucosal pathogens. In keeping with this dual role in tolerance and responsiveness, gastrointestinal T cells have been characterized as adopting a unique, partially activated memory phenotype that distinguishes them from blood T cells *(1–3)*.

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The gut contains immunological inductive sites, such as Peyer's patches and organized lymphoid aggregates within the intestine, and adjacent mesenteric lymph nodes *(4)*. Antigen-specific T cells that are primed in mucosal inductive sites are evidently preprogrammed to return to the mucosa, due to expression of mucosal homing integrins and chemokine receptors. Intestinal effector cells include two distinct populations: intraepithelial lymphocytes (IEL), which have a cytotoxic phenotype and are primarily CD8+, and lamina propria lymphocytes (LPL), which are mainly CD4+ in healthy individuals.

The gastrointestinal lamina propria is a site of profound CD4+ T-cell depletion during acute HIV-1 infection *(5–8)*, and restoration of these cells following initiation of antiretroviral therapy is delayed and incomplete *(8, 9)*. Despite the importance of this site in HIV-1 infection, little is known of the immune response to HIV-1 in mucosal tissues. To better assess the phenotypic characteristics and effector functions of mucosal T cells, several groups have developed protocols for isolating intestinal lymphocytes, either from surgical resection specimens or from biopsies obtained during endoscopy, colonoscopy or sigmoidoscopy. This chapter presents a simple method for processing biopsy tissue that has been adapted from earlier protocols, which used primarily surgical resection tissue *(10–12)*.

In optimizing this protocol, we compared the efficiency of four different techniques for isolating mucosal mononuclear cells from rectal biopsy tissue: manual disruption with a stainless steel mesh screen, mechanical disruption in a commercial tissue homogenizer, enzymatic digestion using a mixture of collagenase and dispase enzymes, and digestion with collagenase type II alone *(13)*. While the mechanical disruption approach was the most rapid and technically straightforward, collagenase type II digestion yielded the greatest number of viable lymphocytes, generally four- to fivefold greater than that obtained by other methods *(13)*. Both yield and cell viability were enhanced using this approach.

The protocol consists of two steps: first, fresh tissue biopsies are incubated with collagenase, type II; second, viable lymphocytes are separated from enterocytes and debris using density gradient centrifugation. The resulting lymphocyte suspension contains a mixture of intraepithelial and lamina propria T cells, as well as B cells. The suspension may be processed in parallel with peripheral blood mononuclear cells (PBMC), and is suitable for most phenotypic and functional assays, including MHC class I tetramer staining, cell surface phenotyping, and cytokine flow cytometry (CFC) *(13–15)*. If desired, cells also may be polyclonally expanded and cultured, then analyzed by ELISPOT or used as effector cells in bulk ^{51}Cr release assays *(16, 17)*.

2. Materials

2.1. Biopsy Collection

1. Flexible sigmoidoscope equipped with single-use disposable biopsy forceps (*see* **Note 1**).
2. Collection medium: RPMI-1640, supplemented with 15% fetal bovine serum, penicillin/streptomycin and L-glutamine (also referred to as "complete medium").
3. Additional antibiotics: piperacillin-tazobactam (Zosyn®, 500 μg/mL) and amphotericin B (optional) (1.25 μg/mL) (*see* **Note 2**).

2.2. Lymphocyte Isolation from Biopsy Tissue

1. Collagenase type II-S (clostridiopeptidase A from Clostridium histolyticum)
2. Shaking incubator, 37 °C.
3. 30-cm^3 disposable syringes attached to a blunt-ended 16-gauge needle.
4. Sterile plastic strainers, 70 μm mesh.

2.3. Discontinuous Percoll Gradient Centrifugation

1. Isotonic Percoll solution: Mix 1 part 10× PBS (or Hank's Basic Salt Solution, HBSS) with nine parts Percoll. Store at 4 °C.
2. 35% and 60% isotonic Percoll in PBS. For 100 mL of 60% Percoll, mix 60 mL of isotonic Percoll with 40 mL PBS. To prepare 100 mL of 35% Percoll, mix 35 mL of isotonic Percoll with 65 mL PBS. Add a few drops of 0.5% phenol red dye (in PBS) to the 60% Percoll solution. This will permit easy visual differentiation of layers within the discontinuous gradient. Store both solutions at 4 °C.

2.4. Polyclonal Expansion

1. For stimulation, select one of the following:
   a. Phorbol myristate acetate (PMA) and ionomycin, 5 ng/mL and 0.5 μg/mL, respectively, in complete medium *(18)*.
   b. Anti-CD3 monoclonal antibody 12F6, 0.1 μg/mL in complete medium (antibody provided by Dr. Johnson T. Wong, Massachusetts General Hospital). Bispecific antibodies, recognizing either CD3:CD4 or CD3:CD8, are available from the same source *(19, 20)*. The CD3:CD4 antibody stimulates depletion of CD4+ T-cells and expansion of CD8+ T-cells, while the CD3:CD8 antibody has the opposite effect. The bispecific MAbs are also used at 0.1 μg/mL in complete medium.
2. Human recombinant interleukin-2 (hrIL-2), 25,000 U/mL (500 ×), stored in aliquots at −80 °C.
3. Piperacillin-tazobactam (Zosyn®, 500 μg/mL) and amphotericin B (optional) (1.25 μg/mL).

4. Autologous PBMC, irradiated with 3000 cGy.
5. 24-well sterile polystyrene tissue culture plates.

3. Methods

3.1. Sample Collection

1. Biopsy samples are collected using sterile, single-use disposable biopsy forceps and are immediately placed in a sterile 50 mL conical centrifuge tube containing 10 mL of complete medium (*see* **Note 3**).
2. After releasing each tissue piece in the collection medium, rinse biopsy forceps with sterile PBS or distilled water before re-introducing into the patient.
3. At the conclusion of the biopsy procedure, close the collection tube and package according to local and federal shipping requirements. Transport the sample to the laboratory as quickly as possible, maintaining at room temperature (25 °C) during transport (*see* **Note 4**).

3.2. Lymphocyte Isolation from Biopsy Tissue

1. Immediately before each procedure, prepare a fresh solution of collagenase type II consisting of 0.5 mg collagenase/mL in RPMI with 5% FCS. Filter the solution (0. 22 μm) to sterilize. One hundred milliliters will be required for processing up to 20–25 individual tissue biopsies (*see* **Note 3**).
2. In a laminar flow biosafety cabinet, transfer the biopsies from the collection tube to a 70-μm nylon mesh screen. Gently wash the biopsies twice with complete medium. This is easily done by pipetting medium over the biopsies while holding the mesh screen over a waste receptacle or a 50-mL conical centrifuge tube.
3. *First incubation*: After washing, transfer the biopsies to a fresh 50 mL conical tube containing 30 mL collagenase solution. Incubate at 37 °C in a shaking incubator for 30 min.
4. After 30 min, break down tissue pieces by repeatedly (10–12 times) passing them through a 12-cm^3 syringe topped with a blunt-ended 16 gauge needle. This should greatly reduce the size of individual tissue fragments.
5. Strain the resulting cell suspension through a disposable nylon 70 μm strainer to separate free cells from undigested tissue. Collect the cell suspension in a fresh 50 mL conical tube. Label this tube “First Digestion”.
6. *Second incubation*: Return the undigested tissue pieces to a 50-mL conical tube, add fresh collagenase solution (30 mL), and incubate in the shaking 37 °C incubator for 30 min.
7. Immediately remove collagenase from the cells that passed through the strainer (“First Digestion”) by washing twice with PBS or culture medium. This is done by centrifuging

for 5 min in a tabletop centrifuge at 700 × *g*, 25 °C, to pellet the cells. Discard the supernatant. Immediate removal of collagenase helps preserve cell integrity and viability.

8. Resuspend the cell pellet in 40–50 mL PBS. Centrifuge again as in Step 7 and discard the supernatant.
9. Repeat Step 8.
10. After the second wash, resuspend the cell pellet in 6 mL complete medium. Place the tube containing the washed cells, loosely capped to allow gas exchange, in a tissue culture incubator at 37 °C, 5% CO_2.
11. Now return to the tissue pieces incubating in the presence of collagenase. Repeat steps 4 thru 6 for a total of three collagenase treatments. By the end of the third treatment, few tissue fragments will remain and most lymphocytes will have been released (*see* **Note 5**).
12. After the second and third collagenase treatments, follow steps 7–10 in order to remove excess collagenase. After washing, the cells that were isolated during the first, second and third treatments should be pooled such that the final total volume is 18 mL.

3.3. Percoll Gradient Enrichment

1. In 15 mL conical centrifuge tubes, prepare the Percoll gradients by underlaying 4 mL of 35% Percoll with 4 mL of 60% Percoll. Prepare as many tubes as needed for the number of biopsies collected. Each tube will accommodate 6 mL of cell suspension; thus, three tubes will be required for 18 mL of cell suspension. The 60% Percoll solution, which will be the lower solution in the tube, is tinted red in order to readily distinguish it from the 35% Percoll.
2. Refrigerate the gradients at 4 °C for one hour before using.
3. Gently layer 6 mL of cell suspension on top of each gradient. Final volume in each 15 mL conical tube will be 14 mL. Centrifuge at 700 × g for 20 min, 4 °C, *without brake*.
4. Enterocytes are located primarily at the top interface (between media and 35% Percoll). Lymphocytes are located primarily at the lower interface (between 35% and 60% Percoll) (*see* **Note 6**).
5. Place the harvested layers in a sterile, 50-mL conical centrifuge tube. Add PBS to a final volume of 50 mL. Invert the tube several times to mix well, then centrifuge for 10 min, 700 × g, 25 °C (*see* **Note 7**).
6. Discard supernatant and repeat the washing step: resuspend the cell pellet in a small volume of PBS, then add PBS to a final volume of 50 mL. Centrifuge as in step 5.
7. After the second wash, discard supernatant. Resuspend the cell pellet in 5 mL of complete medium. Count viable cells in a hemocytometer using trypan blue (*see* **Note 8**).

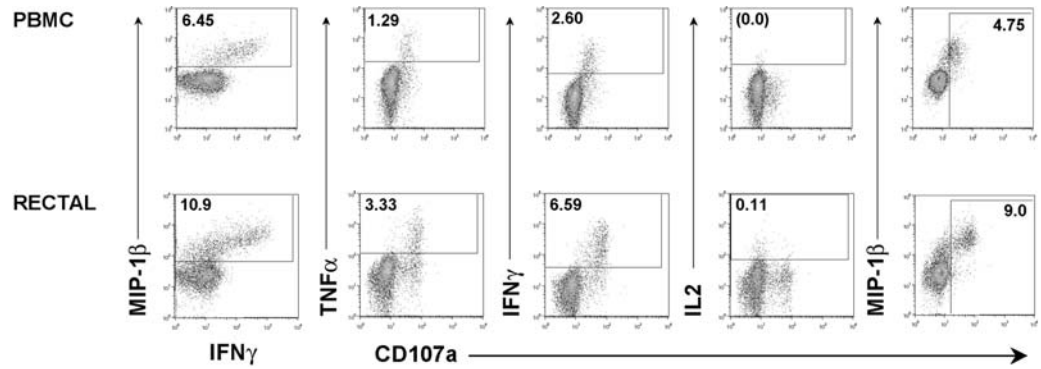

Fig. 23.1. **H**IV-1 gag-specific T-cell responses in rectal mononuclear cells and PBMC. This figure illustrates a standard cytokine flow cytometry (CFC) experiment performed in parallel on freshly isolated PBMC and rectal cells from an HIV-1-positive individual. Cells were stimulated with overlapping 15-mer peptides spanning the entire HIV gag protein and analyzed by nine-color flow cytometry for production of IFNγ, TNFα, IL2, MIP-1β. Degranulation was assessed by staining for CD107a. PBMC are shown on the *top row*; rectal mononuclear cells are shown on the *bottom row*. Numbers indicate percent CD8+ T-cells positive for each marker after subtracting background responses.

8. Adjust the volume as needed to give a final density of $1–2 \times 10^6$ cells/mL. Add piperacillin–tazobactam to a final concentration of 0.5 mg/mL (*see* **Note 2**).
9. Place the cell suspension in the tissue culture incubator at 37 °C, 5% CO_2. Cells may be used immediately or "rested" overnight in the incubator (*see* **Note 9**).
10. **Applications**. Mucosal lymphocytes may be used directly without further manipulation for most flow cytometry staining protocols and cellular immunology assays, including MHC class I tetramer staining, CFC, cell surface phenotyping, magnetic bead sorting, and flow cytometry-based cell sorting (*see* **Notes 10, 11**) (*see* **Fig**. 23.1). For ELISPOT assays, high levels of cytokine secretion preclude analysis of fresh mucosal cells. However, background becomes acceptably low in most cases after polyclonal expansion *(13,16)* (*see* **Note 12**). Polyclonal expansion may also be used to generate bulk cultures that may be tested for cytotoxic function in bulk ^{51}Cr assays *(17)*.

3.4. Polyclonal Expansion

1. Start with $1–2.0 \times 10^6$ cells. Wash and resuspend at a density of $1–2 \times 10^6$/mL in complete medium supplemented with piperacillin-tazobactam (Zosyn®, 500 μg/mL), amphotericin B (optional) (1.25 μg/mL), and hrIL-2 (50 U/mL) (*see* **Note 2**).
2. Add **either** PMA/ionomycin (5 ng/mL and 0.5 μg/mL, respectively) **or** 0.1 μg/mL CD3:4 or CD3:8 bispecific Abs, or anti-CD3 MAb.
3. Three days later, add $5–10 \times 10^6$ autologous irradiated PBMC.

4. Twice weekly, change medium by removing as much supernatant as possible without disturbing the cells. Replace with fresh complete medium supplemented with IL-2 (50 U/mL) and antibiotics. Do not add more PMA or antibody.
5. Every 21–28 days, restimulate cells by adding fresh PMA/ionomycin or antibody.
6. Typically, $0.5–1.0 \times 10^6$ cells are expanded by 10- to 20-fold in 14–21 days using these approaches *(13, 16, 19, 21, 22)* (*see* **Note 12**).

4. Notes

1. Biopsies may be acquired from other sites, including jejunum, ileum, duodenum, and colon. These biopsies are typically 1–3 mm in diameter.
2. Piperacillin-tazobactam (Zosyn®) is particularly efficient at reducing contamination due to intestinal flora *(13)*. Amphotericin B is useful to prevent overgrowth of yeast, but may also retard growth of human cells.
3. The number of biopsies obtained will vary depending upon the site being biopsied and the experimental protocol. Individual research studies will have different protocols for collection of biopsy tissue. Details of any research protocols involving human subjects are subject to review by Institutional Review Boards of participating institutions. Research protocols involving human subjects should always be planned in consultation with collaborating clinicians and local regulatory authorities. Similarly, the details of any protocols involving nonhuman primates are subject to approval of Institutional Animal Care and Use Committees and should be worked out in consultation with veterinary clinicians.
4. It should be stressed that specimen collection and transport issues can dramatically affect cell yield. For best results, biopsy specimens should be transported to the laboratory immediately upon collection, and processed within 2–3 h. Delayed sample processing may greatly reduce cell yield, viability, and assay results. Overnight shipping of specimens is not recommended.
5. Particularly small biopsies may require only two collagenase incubations.
6. Depending on cell yield, the layers may be difficult to see; when in doubt, harvest the interface even if a distinct cell population is not visible. Although the upper Percoll interface should contain mostly epithelial cells, lymphocytes are frequently present as well. Yield can therefore be increased by combining the two layers. A triple gradient, consisting of

20–44–67% Percoll layers, may provide improved separation of epithelial cells and intestinal lymphocytes; in this configuration, enterocytes are found at the interface between 20% and 44% Percoll, and lymphocytes are enriched between the 44% and 67% Percoll layers *(18, 23)*. The presence of epithelial cells is generally not problematic for flow cytometry-based assays, since gating of T-cells is based on size, granularity and expression of lineage-specific surface antigens. However, if desired, epithelial cells may be selectively removed using magnetic beads coated with monoclonal antibody BerEP4 *(18)*.

7. It is important to wash in a large volume of PBS (45–50 mL) in order to dilute the Percoll, allowing cells to pellet.
8. Based upon our experience with human rectal tissue, the yield may vary from approximately 0.2 to 1×10^6 viable lymphocytes per biopsy piece (1–3 mm each). Variation may be caused by a number of factors, including the size of the biopsy forceps, patient-to-patient variation, and details of the isolation procedure.
9. Due to the length of time required to obtain and process primary tissue specimens, it is often convenient to "rest" cells in the incubator overnight before proceeding with experiments. We have found this procedure to be satisfactory in most cases; however, some loss of viability is to be expected.
10. Care should be used when designing flow cytometry experiments using cells that have been subjected to enzymatic digestion protocols. Some surface antigens are adversely affected, resulting in loss or modification of critical epitopes that allow detection using monoclonal antibodies. Collagenase is generally less detrimental to surface antigens than other enzymes such as dispase and pancreatin. Nevertheless, before undertaking these experiments, it is prudent to test all flow cytometry panels in parallel with untreated PBMC and PBMC that have been incubated with collagenase.
11. It is not practical to isolate IEL and LPL separately from small biopsies; however, if purified populations are desired, larger tissue pieces may be obtained from intestinal resection or gastric bypass surgery. IEL are then isolated by rapid shaking of tissue fragments at 37 °C in a solution of 0.75 mM ethylenediamine tetraacetic acid in HBSS. LPL are subsequently isolated by collagenase treatment (*10–12*).
12. Polyclonal expansion can modify activation status and expression of certain phenotypic markers *(13, 17)*. For this reason, freshly isolated cells are preferred for most cellular immunology assays. However, polyclonal expansion provides the advantage of increased cell numbers. In addition, for assays requiring a qualitative or semiquantitative readout, these differences may not be significant. Thus, this approach may

provide an appealing option in cases where cell number is limiting *(21)*. Before using polyclonally expanded cells for a particular application, the performance of fresh and expanded populations should be directly compared.

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