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Isolation and cytokine analysis of lamina propria lymphocytes from mucosal biopsies of the human colon

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ABSTRACT

Much of our understanding of gut-microbial interactions has come from mouse models. Intestinal immunity is complex and a combination of host genetics and environmental factors play a significant role in regulating intestinal immunity. Due to this complexity, no mouse model to date gives a complete and accurate representation of human intestinal diseases, such as inflammatory bowel diseases. However, intestinal tissue from patients undergoing bowel resection reflects a condition of severe disease that has failed treatment; hence a more dynamic perspective of varying inflammatory states in IBD could be obtained through the analyses of pinch biopsy material. Here we describe our protocol for analyzing mucosal pinch biopsies collected predominantly during colonoscopies. We have optimized flow cytometry panels to analyze up to 8 cytokines produced by CD4+ and CD8+ cells, as well as for characterizing nuclear proteins and transcription factors such as Ki67 and Foxp3. Furthermore, we have optimized approaches to analyze the production of cytokines, including TGF-beta from direct *ex vivo* cultures of pinch biopsies and LPMCs isolated from biopsies. These approaches are part of our workflow to try and understand the role of the gut microbiota in complex and dynamic human intestinal diseases.

1. Introduction

Ulcerative colitis and Crohn's disease are the two conditions that comprise inflammatory bowel diseases. Collectively they affect approximately 1.4 million people in North America alone with prevalence on the increase (Lakatos, 2006; Loftus, 2004). IBD is a complex disease and there is a poor understanding of its etiology. Host genetics and immune responses combine somehow with environmental factors to contribute toward the onset of IBD (Sartor, 2006). Genetically susceptible individuals eventually mount an aberrant immune response against intestinal flora and/or dietary antigens causing the archetypal pathology associated with IBD (Duchmann et al., 1995; Maul and Duchmann, 2008; Mow et al., 2004). Activated CD4+ T helper cells in the lamina propria and epithelium of the gut mucosa are key mediators of intestinal inflammation (Funderburg et al., 2013; Zenewicz et al., 2009) and we are performing in-depth analysis of their cytokine production to draw comparisons between active and inactive disease states. (Leung et al., 2014)

Mouse models of IBD have improved our understanding of intestinal immunity but none are a perfect representation of the human diseases (Jones-Hall and Grisham, 2014; Kuhn et al., 1993; Ostanin et al., 2009; Okayasu et al., 1990). Characterization of human samples from both diseased and healthy tissues is critical for our understanding of human intestinal immunity. Unlike mouse experiments, where the entire length of the colon can be dissected, human tissue samples are difficult to

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obtain and can be much more scarce. Analysis of tissue from the human gastrointestinal tract requires harvesting cells from either surgical specimens or pinch biopsies. While surgical specimens provide larger amount of tissue for greater cell yield, they represent a patient population that has failed treatment and does not provide a dynamic picture of all the disease states in IBD. Pinch biopsies allow us to analyze specific areas of the intestine without surgical intervention and thus, mucosal pinch biopsies can provide researchers with a better picture of varying disease conditions; remission, active and inactive colitis. Furthermore, pinch biopsies allow us to sample a single patient multiple times over the course of months or even years providing valuable longitudinal data. However, the major drawback of working with pinch biopsies is that the amount of tissue obtained is limited. It is, therefore, paramount to optimize protocols to ensure maximum cell yield to allow for accurate analysis without compromising the functional properties of the isolated cells, and also to obtain the maximum amount of information from the isolated cells.

Here we describe our optimized protocol for analyzing pinch biopsies obtained during colonoscopies. We now analyze up to 8 cytokines by flow cytometry, gating on CD4+, CD8+ and CD3+ and CD3-cells in a single panel. We utilize a second panel that allows us to examine nuclear proteins and transcription factors such as Ki67 and Foxp3. Furthermore, we have optimized approaches to analyze the production of cytokines, including TGF-beta from direct *ex vivo* cultures of pinch biopsies and LPMCs isolated from biopsies.

2. Materials and methods

2.1. Isolation of lamina propria mononuclear cells (LPMCs) from biopsy tissue

- **Abbreviations**
  LPMC—lamina propria mononuclear cells, RT—room temperature, DMSO—dimethyl sulfoxide
  PMA—phorbol 12-myristate 13-acetate
- Materials
  Supplies:

Item	Source
15 mL conical tube	BD Falcon
50 mL conical tube	BD Falcon
100 μm cell strainer	BD Falcon
1 mL syringe	BD Falcon
3 mL transfer pipet	BD Falcon
96-well tissue culture V-bottom plates	Corning

Reagents:

Item	Source
Collagenase VIII	Sigma-Aldrich
DNase	Sigma-Aldrich
Percoll	GE Healthcare
10× PBS	Gibco
1× PBS	Gibco
RPMI-1640 medium with L-glutamine, 500 mL	Mediatech
Heat inactivated fetal bovine serum	BenchMark
100× penicillin/streptomycin/glutamine	Invitrogen
2-Mercaptoethanol	Sigma-Aldrich
Glycerol	Sigma-Aldrich

Solution and media preparation:

Complete RPMI
500 mL RPMI-1640
10% fetal bovine serum
100× penicillin/streptomycin/glutamine
50 μM 2-mercaptoethanol

FACS buffer
500 mL PBS
5 mL fetal bovine serum
0.05% NaN_3

PROCEDURE

Before starting:

- Note that biopsy tissue should be collected in 15 mL conical tubes with complete media.
- Resuspend collagenase VIII powder in RPMI-1640 at a concentration of 100,000 units/mL and resuspend DNase in 10% glycerol at a concentration of 150 mg/mL.

Steps 1–19: Isolation of LPMCs

1. Prepare collagenase-DNase digestion mix with 100 units/mL of collagenase and 150 μg/mL DNase in 10 mL complete RPMI per sample.
2. Transfer 10 mL of the collagenase-DNase digestion mix to a 50 mL conical tube.
3. Transfer biopsy tissue to the collagenase-DNAse digestion mix tube and shake vigorously.
4. Incubate at 37 °C for 1 h.
5. During the 1 h incubation, prepare the 100% Percoll solution (9 parts Percoll, 1 part 10× PBS). Dilute the 100% Percoll solution to a 40% solution and an 80% solution with complete media. Note: By making the Percoll solution at this time, the solutions will then be at RT by the time the Percoll step arrives.
6. Take out the 50 mL conical tube from the incubator and shake vigorously.
7. Place a 100 μm cell strainer over a new 50 mL conical tube.
8. Pour the collagenase-DNase digestion mix through the 100 μm cell strainer.
9. Mash any undigested tissue through the filter using the bottom of a 1 mL syringe.
10. Add 10 mL 1× PBS through the 100 μm cell strainer to wash out any cells stuck on the filter.
11. Spin tube for 10 min at 600 RCF at RT.
12. Aspirate supernatant carefully down to the pellet level.
13. Resuspend pellet in 5 mL of the 40% Percoll solution and pipet up and down to mix. Transfer mix into a new 15 mL conical tube.
14. Very slowly, underlay 5 mL of the 80% Percoll solution below the 40% Percoll solution. Note: Do not pipet out all of the solution from the pipet. Save a small amount of liquid inside the pipet, as pipetting out the complete 5 mL solution will cause bubbles, which will interfere with the interphase.

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