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Research paper

Quantitative comparison of human intestinal mononuclear leukocyte isolation techniques for flow cytometric analyses

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ABSTRACT

Studies on immune cells derived from the human intestine are needed to understand the pathogenesis of gastrointestinal diseases and to develop novel treatment strategies. Isolation techniques to extract these immune cells from intestinal tissue are largely based on murine studies and comparative data on isolation from human intestine is scarce. In this study we evaluated cell yield, viability, and surface-molecule expression on mononuclear leukocytes, comparing three techniques to obtain a single immune cell suspension from human intestine; low concentrations of either the enzymes Collagenase D or Liberase TL, and enzyme-free mechanical dissociation with the Medimachine. Both enzymatic isolation techniques provided a higher cell yield than mechanical dissociation. Expression of surface molecules remained intact after Collagenase D treatment, while Liberase TL digestion resulted in a strong decrease in the expression of the CD4 receptor. Taken together, Collagenase D digestion provides the highest yield of mononuclear cells while keeping surface molecule expression intact.

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1. Introduction

The human intestinal mucosa is a dynamic interface between the body and the environment. The intestinal lumen provides an extensive surface area for the digestion and absorption of essential metabolites (Mowat and Agace, 2014). At the same time, adequate barrier function is required to prevent microbial translocation (Brown et al., 2013; Cebra, 1999). To maintain homeostasis under continuous microbial exposure, tissue-resident intestinal immune cells such as lymphocytes and monocyte-derived cells play a crucial role. However, protective immune responses need to be tightly regulated in order to prevent excess inflammation and collateral tissue damage (Siddiqui and Powrie, 2008; Veenbergen and Samsom, 2012).

Dysregulation of intestinal immune cells leads to increased susceptibility to severe infections, while deficient tolerant responses of lymphocytes and monocyte-derived cells provide the underlying conditions for intestinal inflammatory diseases such as ulcerative colitis (Troncone et al., 2013). In order to understand the pathogenesis of intestinal diseases, in-depth analyses of immune responses at the tissue level are essential as populations of immune cells in the blood and the intestinal compartments differ substantially (Kunkel and Butcher, 2002).

The number of studies investigating tissue-resident immune cells is rapidly increasing (Annunziato et al., 2007; Bunders et al., 2012; Sathaliyawala et al., 2013; Thome et al., 2016). Throughout those studies, various techniques are applied to isolate the mononuclear leukocyte fraction from the human intestine, first pioneered by Wahl and Smith (1991). However, these techniques differ in terms of cell yield and expression of the molecules of interest on the cell surface (Chen et al., 2014; Shen et al., 2015).

Mucosal lymphoid and monocyte-derived cells are present in several intestinal compartments; the epithelium, the underlying lamina propria, the Peyer's patches (PPs) embedded in the small intestine, and the isolated lymphoid follicles (ILFs) embedded in the colorectum. These compartments contain cell types with a phenotype and functionality unique to their anatomical location (Mowat and Agace, 2014). In this study we focus on the isolation of mononuclear leukocytes from the epithelium and from the lamina propria, excluding PPs and ILFs on macroscopic visual inspection of the tissues.

The isolation of the intraepithelial mononuclear leukocytes (IELs) is homogenous among protocols; dithiothreitol (DTT) and ethylenediaminetetraacetic acid (EDTA) are used to remove mucus and to detach the epithelial cells from the tissue. Subsequently, enrichment of the cell suspension for IELs is commonly achieved with gradient centrifugation, which separates cells based on their buoyant density

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(Fuss et al., 2009). However, layer gradients vary between protocols from 60%–67.5% Percoll (Braunstein et al., 1997; Ebert and Roberts, 1995; Lundqvist et al., 1992) to using standard Ficoll-Hypaque (Comer et al., 1986).

After detachment of the epithelial layer, the mononuclear leukocytes from the lamina propria (LPLs) can be obtained via mechanic or enzymatic disaggregation of the tissue. The described isolation techniques to obtain this cell population vary greatly with regards to cell yield (Shacklett et al., 2003). Furthermore, the expression of molecules of interest on the cell surface (Chen et al., 2014; Shen et al., 2015) can be differentially altered by the techniques thereby impacting the feasibility of the study as well as the interpretation of the obtained results. An isolation technique that harvests the maximal number of cells while limiting alterations to cell surface marker expression is particularly relevant to pediatric studies as collection of sufficient tissue is often more challenging than in adults.

Taken together, comparative data of cell numbers and surface marker consistency of the most frequently used techniques to isolate mononuclear leukocytes from the human intestinal epithelium and lamina propria is needed to design the appropriate methodology for performing intestinal immune cell analyses. In this study, we compared three frequently used methods of lamina propria disaggregation to obtain a single mononuclear leukocyte suspension: Medimachine (enzyme-free mechanical disaggregation), and low concentrations of the enzymes Collagenase D and Liberase TL. Furthermore, we investigated the efficiency of using different density gradients to enrich for IELs and LPLs. The different isolation and enrichment techniques of mononuclear cells (T lymphocytes and monocyte-derived cells) from intestinal tissue were compared regarding cell yield, viability, and surface marker expression.

2. Materials and methods

2.1. Tissue samples

Human tissues were collected after the donors or their guardians provided informed consent. All experiments were performed on fresh large bowel tissue from ten individuals obtained during surgery (Supplementary Table 1); four were infants (median age 6 months, interquartile range (IQR) 4–11 months), of which three were surgically treated for Hirschsprung's disease and one for anorectal malformation; six were adults (median known age 62 years, IQR 44–63 years), of which four were surgically treated for ulcerative colitis and two for colorectal cancer. All tissue was derived from the surgical margin and based on visual assessment all tissues, except from one adult patient with ulcerative colitis, were presumed to be not severely affected. Every tissue was processed within 6 h after resection. Because of the variation in donor age and diagnosis, direct comparisons were only made within individual donors, not between them. The study was approved by the medical ethical committee of our institute, the Academic Medical Center (University of Amsterdam) and in accordance with the Declaration of Helsinki.

2.2. Tissue preparation

The tissue was rinsed by manually shaking 20 s in 20 ml sterile PBS in a 50 ml canonical centrifuge tube (Greiner, Sigma-Aldrich), followed by removal of the muscular layer using scissors (Fig. 1). To determine cell yield per cm^2, the size of the mucosal tissue was measured after removal of the muscular layer. The tissue was divided into pieces with equal surface area (0.5 cm^2).

2.3. Epithelial layer detachment

To remove the remaining mucus and detach the epithelial layer containing the IELs, the intestinal fragments were incubated for 20 min in a shaking water bath (approximately 100 strokes/min) at 37 °C in 10 ml Iscove's Modified Dulbecco's Medium (IMDM; Lonza, Verviers, Belgium) containing 2 mM DTT, 5 mM EDTA, 5% fetal calf serum (FCS; Biological Industries, Kibbutz Beit Haemek, Israel), 100 U/ml Penicillin, and 100 μg/ml Streptomycin (Gibco, Life Technologies) in a 50 ml canonical centrifuge tube. The suspension including the tissue was then vortexed (IKA; MS3 basic Lab Shaker) at maximum speed (3000 rpm for this model) for 15 s after which the cell suspension without tissue was passed through a 70 μm single-cell strainer (Falcon, Corning, USA), rinsed with 4 °C PBS and centrifuged at 400 G at 4 °C for 10 min. A second incubation with the above described DTT/EDTA solution followed for the remaining tissue using the same procedure and resulting in a second single cell suspension. The two single cell suspensions were pooled after washing them with 4 °C PBS and stored in PBS on ice until density gradient centrifugation.

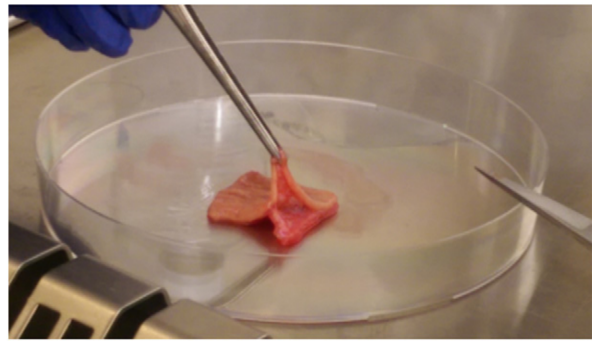

Fig. 1. Removal of the muscular layer (bottom) from the mucosa (top). Tissue from a pediatric donor is shown.

2.4. Lamina propria disaggregation

2.4.1. Mechanical disaggregation using the Medimachine

The BD Medimachine System (BD Biosciences) was used as described before (Bunders et al., 2012). Briefly, it is an automated mechanical disaggregation system of solid human tissues without enzymes. The Medimachine spins the tissue in the Medicon filter (BD Biosciences, San Jose, USA) to disaggregate tissue fragments into single cells. After detachment of the epithelial layer, one tissue fragment at a time (0.5 cm^2) was placed in the Medicon filter with 1 ml PBS. The single-cell suspension was obtained by placing a 5 ml syringe on the out-port of the Medimachine and extracting the cell suspension. This procedure was repeated until each fragment was dissolved completely. Cells were passed through a 70 μm single-cell strainer, rinsed with 4 °C PBS, and centrifuged at 400 G (4 °C) for 10 min. The obtained single cell suspension was stored in PBS on ice until density gradient centrifugation. Medimachine isolation was always compared with Collagenase D isolation; half of the tissue of one donor was mechanically disaggregated with Medimachine while the other half was enzymatically digested with Collagenase D as described in the next paragraph (and in Fig. 2).

2.4.2. Enzymatic disaggregation using Collagenase D

After detachment of the epithelial layer, tissue fragments were minced with scissors and placed in a 50 ml tube with 10 ml disaggregation solution: IMDM with 1 mg/ml (0.15 U/mg) Collagenase D (Roche, Mannheim, Germany), 1% FCS, and 1000 U/ml DNAse type I (Roche). The fragments were incubated in the medium in a shaking water bath at 37 °C for 30 min (approximately 100 strokes/min). The supernatant containing the cell suspension was collected and passed through a 70 μm single-cell strainer, rinsed with 4 °C PBS, and centrifuged at 400 G at 4 °C for 10 min. The remaining fragments were incubated in fresh disaggregation solution containing Collagenase D for another 30 min after which the remaining cell suspension was filtered through a 70 μm single-cell strainer. The filtered single cell suspensions were

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