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Research paper

Optimization of murine small intestine leukocyte isolation for global immune phenotype analysis

Andrew W. Goodyear [a,1], Ajay Kumar [a,1], Steven Dow [a,2], Elizabeth P. Ryan [a,b,*]

[a] Department of Clinical Sciences, Colorado State University, 300 W. Drake Street, Fort Collins, CO 80523, USA
[b] Department of Environmental and Radiological Health Sciences, Colorado State University, 200 W. Lake Street, Fort Collins, CO 80523, USA

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ABSTRACT

New efforts to understand complex interactions between diet, gut microbiota, and intestinal immunity emphasize the need for a standardized murine protocol that has been optimized for the isolation of lamina propria immune cells. In this study multiple mouse strains including BALB/c, 129S6/Sv/EvTac and ICR mice were utilized to develop an optimal protocol for global analysis of lamina propria leukocytes. Incubation temperature was found to significantly improve epithelial cell removal, while changes in media formulation had minor effects. Tissue weight was an effective method for normalization of solution volumes and incubation times. Collagenase digestion in combination with thermolysin was identified as the optimal method for release of leukocytes from tissues and global immunophenotyping, based on the criteria of minimizing marker cleavage, improving cell viability, and reagent cost. The effects of collagenase in combination with dispase or thermolysin on individual cell surface markers revealed diverse marker specific effects. Aggressive formulations cleaved CD8α, CD138, and B220 from the cell surface, and resulted in relatively higher expression levels of CD3, γδ TCR, CD5, DX5, Ly6C, CD11b, CD11c, MHC-II and CD45. Improved collagenase digestion significantly improved viability and reduced debris formation, eliminating the need for density gradient purification. Finally, we demonstrate that two different digestion protocols yield significant differences in detection of $CD4^+$ and $CD8^+$ T cells, NK cells, monocytes and interdigitating DC (iDC) populations, highlighting the importance and impact of cell collection protocols on assay outputs. The optimized protocol described herein will help assure the reproducibility and robustness of global assessment of lamina propria immune responses. Moreover, this technique may be applied to isolation of leukocytes from the entire gastrointestinal tract.

1. Introduction

The gastrointestinal mucosal environment is becoming increasingly interrogated for its dynamic properties of high immunological importance, and with regard to heightened knowledge and roles for the gut microbiome to interface with diet and gut associated inflammatory diseases (Hooper et al., 2012; Veldhoen and Brucklacher-Waldert, 2012). While immune cell isolation protocols typically follow the general steps of mucus removal, epithelial cell removal, and collagenase digestion; there have been limited efforts since the 1980s to improve protocols for global murine intestinal immune phenotypic analysis (Castro et al., 1974; Davies and Parrott, 1981; Leventon et al., 1983; Dillon and MacDonald, 1985). Further complicating isolation methods is the precise development of protocols within inbred mouse strains, and multiple modifications aimed at isolation of specific cell types

* Corresponding author at: Department of Environmental and Radiological Health Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, 200 W. Lake Street, 1680 Campus Delivery, Fort Collins, CO 80523-1680, USA. Tel.: +1 970 491 1536.
E-mail addresses: goody13@colostate.edu (A.W. Goodyear), Ajay.Kumar@colostate.edu (A. Kumar), sdow@colostate.edu (S. Dow), e.p.ryan@colostate.edu (E.P. Ryan).
[1] Tel.: +1 970 491 2100.
[2] Tel.: +1 970 492 4382.

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such as B cells, γδ T cells and dendritic cells (Van der Heijden and Stok, 1987; Ye et al., 2010; Geem et al., 2012). For instance, certain studies do not utilize mucolytic agents such as dithiothreitol (DTT) while other studies utilize concentrations between 1 and 5 mM (Davies and Parrott, 1981; Waidmann et al., 2002; Resendiz-Albor et al., 2005). Removal of epithelial cells has been performed by mechanical disruption, enzymatic removal, and with chelating agents such as ethylenediaminetetraacetic acid (EDTA)(Castro et al., 1974; Davies and Parrott, 1981; Sheridan and Lefrancois, 2012). The use of EDTA appears to be the most rapid and common method, though EDTA concentrations ranging from 1 to 30 mM have been reported (Ericsson and Agace, 2004; Resendiz-Albor et al., 2005). In addition, incubations have been performed at room temperature (RT) or 37 °C with frequency and duration combinations varying widely from one wash for 90 min to 5 min washes for a total of 30 min (Davies and Parrott, 1981; Ericsson and Agace, 2004). Although expected cell yields and phenotype results are typically reported, changes to media formulation are generally not justified in the reported results. Therefore, identifying optimal conditions for recovery of intestinal leukocyte populations under diverse conditions of intestinal inflammation and anatomic location is a continuous challenge.

Use of different collagenase formulations for enzymatic digestion also contributes to protocol variability. While some protocols have used purified collagenase such as CLSPA from Worthington, or Liberase formulations from Roche (Davies and Parrott, 1981; Foureau et al., 2010), the vast majority have utilized crude collagenase preparations such as Type I, II, VI or VIII from Sigma-Aldrich, type III from Worthington, or collagenase D from Roche (Crofton et al., 1978; Lyscom and Brueton, 1982; Tseng, 1982; Lycke, 1986; Waidmann et al., 2002; Fujimoto et al., 2011). Justification for the choice of collagenase utilized has been rarely provided. Furthermore, optimal conditions for digestion of intestinal tissue are not reported by the manufacturer (Sigma-Aldrich, 2013). In addition, the use of crude collagenase preparations complicates protocol optimization because, as reported previously, the lot to lot variability necessitates re-optimization (Van der Heijden and Stok, 1987). Moreover, contaminating proteases may vary between lots resulting in alternative cleavage of surface markers (personal communication, Roche technical support). Finally, digestion conditions vary widely and include digestion in Hank's balanced salt solution (HBSS), or culture media containing 0, 5, 10 or 20% serum, with incubation at RT or 37 °C in atmospheric or 5% CO_2 environments (Cebra et al., 1977; Tseng, 1982; Gautreaux et al., 1994; Johansson-Lindbom et al., 2005; Salazar-Gonzalez et al., 2006; Sheridan and Lefrancois, 2012). To the best of our knowledge, the effects of collagenase formulations on surface markers from intestinal tissue have not been investigated, therefore the data presented herein supports the ability to perform a global immune phenotype analysis of murine gastrointestinal tissue.

The prevalence of gastrointestinal diseases with known and yet undiscovered immune dysfunctions is increasing (e.g. Ulcerative colitis, Crohn's, celiac, cancer, obesity, etc.). These conditions exemplify the strong rationale for identifying experimental variables necessary for efficient processing of intestinal cells that are reproducible and meet high throughput, well powered experimental needs. For dietary intervention and enteric pathogen infection studies performed in our laboratory (Henderson et al., 2012; Kumar et al., 2012), increased sample sizes and the need for global immune phenotypic analysis prompted the protocol optimization described in this manuscript.

2. Methods

2.1. Stock solutions

10× phosphate buffered saline (PBS) was prepared according to the manufacturer's instructions (Calbiochem, Billerica, MA), and was stored at 4 °C. Antibiotic stocks included penicillin/streptomycin which was purchased as a 10,000 unit/ml penicillin and 10 mg/ml streptomycin stock from Life Technologies (Carlsbad, CA) and aliquots were stored at −20 °C. Enrofloxacin powder (Sigma-Aldrich, St. Louis, MO) was dissolved at 250 mg/ml in 10% acetic acid (Sigma-Aldrich), filter sterilized, and aliquots were frozen at −80 °C. Upon thawing enrofloxacin stocks were diluted to 25 mg/ml with sterile 60 mM Tris base (Fisher Scientific, Pittsburgh, PA). Finally, polymyxin B (Sigma-Aldrich) was dissolved in distilled water at 10^5 units/ml, filter sterilized, and aliquots stored at −80 °C. All antibiotic stocks were used within 6 months of preparation based on previously published stability studies (Griffith and Bodily, 1992; Okerman et al., 2007; Goodyear et al., 2013). 10× HBSS was purchased from Sigma-Aldrich and stored at RT. A 7.5% sodium bicarbonate (Sigma-Aldrich) stock solution of was prepared in dH_2O, sterile filtered and stored at RT. 1× HBSS was prepared by diluting 10× HBSS ten-fold with dH_2O, adding 4.2 mM $NaHCO_3$ and adjusting the pH to 7.4 (HBSS). HBSS was stored at 4 °C and used within 6 months. 1 M DTT (Amresco, Solon, OH) stocks were prepared in dH_2O, filter sterilized and stored at −20 °C. DTT stocks were used within one year, and discarded after two freeze/thaw cycles. Upon thawing DTT stocks were used immediately and either re-frozen or discarded as DTT is highly unstable at RT or 4 °C (Nealon and Henderson, 1977). 0.5 M EDTA pH 7.2–7.3 was prepared by dissolving EDTA (MP Biomedicals, Solon, OH) in dH_20 and adjusting the pH with 10 N sodium hydroxide (Fisher Scientific). 1 M HEPES (Fisher Scientific) was prepared in dH_2O, the pH was adjusted to 7.4 and was stored at RT. Liberase formulations from Roche contained a standard amount of purified collagenase in combination with dispase (D) or thermolysin (T), at low (DL, TL), medium (TM) or high (DH, TH) concentrations. Liberase stocks were prepared by re-suspending the lyophilized power in sterile HBSS at 13 Wünsch units/ml, aliquots were stored at −20 °C. Collagenase D (Sigma-Aldrich) stocks were prepared by dissolving the lyophilized powder in HBSS at 5 mg/ml, and aliquots were frozen at −20 °C. Glycerol stocks of DNase I from bovine pancreas (Sigma-Aldrich; Cat: D5025) were prepared. DNase I was dissolved at 10^4 Kunitz/ml in 10 mM Tris pH 7.4, 10 mM $CaCl_2$, 10 mM $MgCl_2$, and 50% glycerol, all reagents from Fisher Scientific. DNase I glycerol stocks were stored at −20 °C, used within 1 year, and stored on ice whenever not at −20 °C. Trypsin inhibitor (Sigma-Aldrich) was dissolved in PBS at 10 mg/ml and aliquots were stored at −20 °C.