



Research paper

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



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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 CD8 α , CD138, and B220 from the cell surface, and resulted in relatively higher expression levels of CD3, $\gamma\delta$ 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.

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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

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such as B cells, $\gamma\delta$ 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% CO₂ 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 10⁵ 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 dH₂O, sterile filtered and stored at RT. 1× HBSS was prepared by diluting 10× HBSS ten-fold with dH₂O, adding 4.2 mM NaHCO₃ 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 dH₂O, 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 dH₂O and adjusting the pH with 10 N sodium hydroxide (Fisher Scientific). 1 M HEPES (Fisher Scientific) was prepared in dH₂O, 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 resuspending the lyophilized powder 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 10⁴ Kunitz/ml in 10 mM Tris pH 7.4, 10 mM CaCl₂, 10 mM MgCl₂, 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.

2.2. Working solutions

1× PBS was prepared by diluting 10× PBS in dH₂O. In an attempt to prevent non-specific activation of immune cells by enteric microflora or experimental infection all solutions were supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin to control extracellular bacteria, 25 µg/ml enrofloxacin to control intracellular bacteria, and 100 units/ml polymyxin B to neutralize LPS (PSEPx). Complete RPMI-1640 (cRPMI) was prepared utilizing RPMI-1640 media (HyClone, Logan, UT) and adding 10% fetal bovine serum (Atlas Biologicals, Fort Collins, CO), 2 mM L-glutamine (Life Technologies, Carlsbad, CA), 1 mM sodium pyruvate (Life Technologies), 1× non-essential amino acids (Life Technologies), and PSEPx. Solution 1 was used to remove mucus and residual intestinal contents and consisted of HBSS + PSEPx supplemented with 2% FBS, and 5 mM DTT was added immediately before use from 1 M DTT freezer stocks. Any remaining solution 1 was discarded due to the unstable nature of DTT (Nealon and Henderson, 1977). Solution 2 was utilized for epithelial cell removal and was composed of HBSS + PSEPx containing 2% FBS and 5 mM EDTA. Solution 3 was utilized for collagenase digestion and consisted of HBSS + PSEPx supplemented with 10 mM HEPES. 20× Liberase TM stocks included 4 Wünsch unit/ml and 4000 Kunitz/ml DNase prepared in solution 3. 20× stock was ultimately diluted to a final working concentration of 0.2 Wünsch units/ml Liberase TM, and 200 Kuntz units/ml DNase in solution 3. Working solutions of collagenase D were prepared in HBSS and contained 40 collagen units/ml collagenase D, 30 Kuntz units/ml DNase, and 250 µg/ml trypsin inhibitor.

2.3. Mice

BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME), 129S6/SvEvTac mice from Taconic Laboratories (Germantown, NY), and institute for cancer research (ICR) mice from Harlan (Indianapolis, IN). BALB/c and 129S6/SvEvTac were female, and ICR mice were male or female. Mice were 8 to 40 weeks of age upon euthanasia. Mice were housed under pathogen-free conditions with filtered air. All experiments involving animals were approved by the Institutional Animal Care and Use Committee at Colorado State University.

2.4. Organ harvest, removal of intestinal contents, and tissue processing

Following euthanasia, the small intestine tissue was harvested, and mesentery and adipose tissue was removed. The tissue was arranged in a “spaced swiss roll” orientation within a 100 × 15 mm Petri dish (VWR International, West Chester, PA), and placed on ice. Next, rather than flushing out intestinal contents with increased pressure, the intestine was inflated with cold PBS to solubilize intestinal contents and grossly visible Peyer's patches were removed. The tissue was then cut open longitudinally and drawn through a pair of curved forceps while applying gentle pressure to remove intestinal contents. The processed tissue was then weighed and this weight was utilized to determine volumes and incubation times for all subsequent steps. Next the tissue was cut into 2–4 cm lengths, placed in a 50 ml conical tube containing 10 ml cRPMI and stored on ice. If

desired, intestinal contents were then stored at the appropriate temperature for future analysis.

2.5. Mucus removal and epithelium isolation

2.5.1. Optimized protocol

Washes were performed in polypropylene 50 ml conical tubes (VWR international). Tubes were sealed, laid flat on a platform shaker and taped to the platform. All washes were performed with shaking at 200 RPM as this speed resulted in vigorous, but not turbulent mixing. Previous studies demonstrated that visual observation of solution cloudiness by microscopy was an accurate assessment of epithelial cell removal (Bull and Bookman, 1977; Davies and Parrott, 1981; Tagliabue et al., 1982), and this technique was applied herein. In preliminary studies, 10 ml EDTA washes were found to saturate in about 10 min (data not shown). Therefore, 10 min washes were utilized for initial optimization studies. In experiments where incubations were performed at 4 °C or 37 °C, solutions were acclimated to the appropriate temperature prior to addition. Incubations performed at 4 °C were performed by adding reagents cooled to 4 °C and shaking at RT. Platform shakers included, for RT (MaxQMini 4000, Thermo Scientific, Waltham, MA) and 37 °C incubations (Forma Orbital Shaker Model 420, Thermo Scientific). Tissue weight was used to determine the solution volume and incubation times for all solutions (see Results). For all wash solutions to be discarded as waste, samples were poured through a 3 inch diameter food strainer (Walmart, Bentonville, AR) to rapidly re-isolate tissue. For isolation of epithelial cells from EDTA washes samples were filtered through a 70 micron cell strainer (BD Biosciences, San Jose, CA). Epithelial samples were stored on ice and cells from all EDTA washes were pooled. If necessary, cell strainers were rinsed with dH₂O between washes to remove debris. Epithelial cells were then centrifuged at 300 × g for 5 min at 8 °C, washed twice in 5 ml cold HBSS + 2% FBS, resuspended in 5 ml cRPMI + PSEPx and stored on ice.

2.5.2. Previous protocol

Intestinal tissue was flushed with PBS, washed 4 × 3 min in PBS (25 ml per sample) at RT and 2 × 10 min in HBSS + 5 mM DTT (25 ml per sample) at RT. Next tissues were washed 2 × 15 min in HBSS + 30 mM EDTA (25 ml per sample) at RT to remove epithelial cells. Isolation solutions contained penicillin and streptomycin at concentrations described in Section 2.2.

2.6. Collagenase digestion

2.6.1. Optimized protocol

Following epithelial cell removal tissues were transferred into 10 ml solution 3 to remove residual FBS and EDTA as both of these have been shown to inhibit collagenase (Hook et al., 1971; McShane et al., 1989; Roche, 2013). Samples were shaken at 200 RPM at 37 °C for 10 min and the solution was discarded as waste. Tissue samples were then transferred to 60 × 15 mm Petri dishes (Greiner Bio-One, Monroe, NC) containing solution 3 (HBSS + 10 mM HEPES). Volumes of solution 3, Liberase TM solution and incubation times were determined based on tissue weight (see Results). Tissues were then minced into fine pieces and after all samples were

minced the correct volume of $20\times$ Liberase TM solution was added to obtain 0.2 Wünsch units/ml Liberase TM and 200 Kunitz/ml DNase. Samples were digested for 30 min at 37°C under atmospheric CO_2 levels, and mixed every 10 min. The digestion was performed in serum free HBSS because the digestion was shortened to 30 min, and because serum has been shown to inhibit collagenase (Roche, 2013). HEPES buffer was added to maintain a stable pH while tissues are minced. Finally, samples were digested under atmospheric CO_2 due to the sodium bicarbonate concentration used in HBSS. For samples requiring less than 5 ml total volume, the digestion was performed in 14 ml round bottom snap cap tubes (BD Biosciences) with shaking at 200 RPM at 37°C for 30 min. Following digestion, 0.5 volumes cRPMI + PSEPx was added to stop the collagenase. Samples were triturated $3\times$ through an 18 gauge needle, and filtered through a 70 micron cell strainer (BD Biosciences). Cells were pelleted by centrifugation at $300\times g$ for 5 min at 8°C , washed twice in 5 ml GI-HBSS + 2% FBS, resuspended in 5 ml cRPMI + PSEPx and stored on ice.

To investigate the effects of collagenase formulations on different surface markers, the percent of total cells and mean fluorescent intensity (MFI) for 20 distinct surface markers was investigated. The goal was to identify a formulation which sufficiently digested the tissue while minimizing marker cleavage. Therefore, we identified the formulation resulting in the strongest and weakest signal for each marker based on the MFI or the mean percent of total cells. The frequency of the total number of strongest and weakest marker expression results were determined for each Liberase formulation and presented as a ratio. This ratio was used to determine which formulation was optimal on a global scale, with a higher ratio being advantageous.

2.6.2. Previous protocol

Tissues were washed in HBSS for 5 min and transferred to collagenase digestion solution. Samples were digested in 40 collagen units/ml collagenase D (Sigma-Aldrich units) + 30 Kunitz/ml DNase I and 250 $\mu\text{g}/\text{ml}$ trypsin inhibitor in cRPMI supplemented with 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (10 ml per sample). Digestion was performed in a 100×15 mm Petri dish at 37°C + 5% CO_2 for 1 h and 30 min, and mixed every 30 min. Following digestion samples were mechanically disrupted through a 70 micron cell strainer, pelleted by centrifugation and washed twice in HBSS. Samples were then purified by density gradient centrifugation as described in Section 2.7.

2.7. Density gradient centrifugation

OptiPrep (60% Iodixanol solution) (Sigma-Aldrich) was diluted to 1.09 g/ml in 0.8% NaCl (Fisher Scientific) containing 5 mM EDTA (MP Biomedicals) and 10 mM Tricine (Sigma-Aldrich), pH 7.4. Following collagenase digestion samples were resuspended in cRPMI + PSEPx and overlaid over 1.09 g/ml OptiPrep. Samples were centrifuged at $1700\times g$ for 10 min with no brake based on previously reports (Gautreaux et al., 1994; Axis, 2013). Following centrifugation the cell layer on top of the 1.09 g/ml OptiPrep solution was transferred to a new tube and cells were washed

twice with HBSS + 2% FBS. Samples were then re-suspended in cRPMI and stored on ice until analysis.

2.8. Cell viability

Cell viability was determined by exclusion of 0.4% trypan blue (HyClone, Logan, UT) and counting on a hemocytometer. In separate studies live/dead analysis was performed by flow cytometry. 1 μM SYTO-9 (Life Technologies) and 10 μM propidium iodide (Sigma-Aldrich) were added to samples in FACS buffer. Samples were incubated for 5 min at RT and immediately analyzed by flow cytometry as described in Section 2.9. Cells were defined as SYTO-9⁺ events, live cells as SYTO-9⁺/PI⁻, dead cells as SYTO-9⁺/PI⁺, and debris as SYTO-9⁻ events.

2.9. Flow cytometry

Flow cytometry was performed as described previously (Goodyear et al., 2010). Briefly, directly conjugated antibodies were purchased from eBioscience (San Diego, CA), BD Biosciences, or BioLegend (San Diego, CA). Antibodies from eBioscience included anti-CD3 (Alexa Fluor 488 conjugated; clone 145-2C11), anti-PDCA (PE conjugated; clone eBio927), anti-CD117 (PE conjugated; clone 2B8), anti-CD11c (PE-Cy7 conjugated; clone N418), anti-CD5 (PE-Cy7 conjugated; clone 53-7.3), anti-CD103 (APC conjugated; clone 2E7), anti-Fc ϵ R1 α (APC conjugated; clone MAR-1), anti-CD11b (Alexa Fluor 700 conjugated; clone M1/70), anti-CD8 α (Alexa Fluor 700 conjugated; clone 53-6.7), anti-MHC-II (APC-eF780 conjugated; clone M5/114.15.2), anti-B220 (APC-eF780 conjugated; clone RA3-6B2), anti-F4/80 (eF450 conjugated; clone BM8), anti-CD4 (eF450 conjugated RM4-5), anti-CD45 (biotin conjugated; clone 30-F11). From BD Biosciences anti-Ly6G (FITC conjugated; clone 1A8), anti- $\gamma\delta$ TCR (FITC conjugated; clone GL3), anti-CD138 (PE conjugated; clone 281-2), anti-Siglec-F (PE-CF594 conjugated; clone E50-2440), anti-CD3 (PE-CF594 conjugated; clone 145-2C11), anti Ly6C (PerCP-Cy5.5 conjugated; clone AL-21). Finally, anti-DX5 (PerCP-Cy5.5 conjugated; clone DX5) was purchased from BioLegend. Single cell suspensions were washed in FACS buffer (PBS + 2% FBS + 5 mM sodium azide (Fisher Scientific)). Prior to staining, samples were incubated with FACS block consisting of 50 mg/ml normal mouse serum (Jackson ImmunoResearch, West Grove, PA), 200 $\mu\text{g}/\text{ml}$ human IgG (Jackson ImmunoResearch) and 5 $\mu\text{g}/\text{ml}$ unlabeled anti-mouse CD16/32 (clone 93, eBioscience) for 5 min at RT to block against nonspecific antibody binding. Antibodies were diluted 1/200 in FACS buffer and incubated with samples for 30 min on ice, followed by washing with FACS buffer. For biotinylated antibodies, streptavidin-pacific orange (Life Technologies) was added at a 1/500 dilution for 20 min on ice. After washing in FACS buffer, cells were fixed in 1% paraformaldehyde (Electron Microscopy Science, Hatfield, PA) in PBS for 15 min at 4°C , washed, resuspended in FACS buffer and stored at 4°C until analyzed. Flow cytometry was performed using a Gallios flow cytometer using Gallios software version 1.2 (Beckman Coulter, Fullerton, CA). Analysis was performed using FlowJo software version 7.6.5 (Tree Star Inc., Ashland, OR). Unless noted, cell population definitions utilized throughout this report are listed in Table 1.

Table 1
Population definitions.

Population	Definition
T cells	FSC-SSC ^{low} /DX5 ⁻ /CD3 ⁺
CD4 ⁺ T cells	FSC-SSC ^{low} /DX5 ⁻ /CD3 ⁺ /CD4 ⁺
CD8 ⁺ T cells	FSC-SSC ^{low} /DX5 ⁻ /CD3 ⁺ /CD8 α ⁺
$\gamma\delta$ T cells	FSC-SSC ^{low} /CD45 ⁺ / $\gamma\delta$ TCR ⁺
NK cells	FSC-SSC ^{low} /CD3 ⁻ /DX5 ⁺
NK-T cells	FSC-SSC ^{low} /CD3 ⁺ /DX5 ⁺
B cells	FSC-SSC ^{low} /CD45 ⁺ /CD3 ⁻ /DX5 ⁻ /B220 ⁺
B1 B cells	FSC-SSC ^{low} /CD45 ⁺ /CD3 ⁻ /DX5 ⁻ /B220 ⁺ /CD5 ⁺
Plasma cells	FSC-SSC ^{low} /CD45 ⁺ /CD138 ⁺
Allergy (Fc ϵ R1 α ⁺)	FSC-SSC ^{low} /CD45 ⁺ /Fc ϵ R1 α ⁺
Basophils	FSC-SSC ^{low} /CD3 ⁻ /B220 ⁻ /Fc ϵ R1 α ⁺
Mast cells	FSC-SSC ^{low} /CD3 ⁻ /B220 ⁻ /Fc ϵ R1 α ⁺ /CD117 ⁺
Neutrophils	CD45 ⁺ /Siglec-F ⁻ /CD11b ⁺ /Ly6G ⁺
Macrophage	CD45 ⁺ /Siglec-F ⁻ /CD11b ⁺ /Ly6G ⁻ /Ly6C ⁻ /F480 ⁺
Monocyte	CD45 ⁺ /Siglec-F ⁻ /CD11b ⁺ /Ly6G ⁻ /Ly6C ⁺
Interdigitating DC (iDC)	CD45 ⁺ /Siglec-F ⁻ /CD11c ⁺ /MHC-II ⁺ /CD103 ⁺
Myloid DC (mDC)	CD45 ⁺ /Siglec-F ⁻ /CD11c ⁺ /MHC-II ⁺ /CD11b ⁺
Plasmacytoid DC (pDC)	CD45 ⁺ /Siglec-F ⁻ /CD11c ⁺ /MHC-II ⁺ /PDCA ⁺
Eosinophil	CD45 ⁺ /Siglec-F ⁺

2.10. Statistical analysis

Statistical analysis was performed in Graph Pad Prism version 5 software (Graph Pad, San Diego, CA). Analysis between two groups was performed by a two-tailed Student's *t*-test. Comparisons between three or more groups were performed by a one-way ANOVA followed by a Tukey's multiple means test. Correlation analysis was performed by a two-tailed Pearson analysis. Differences were considered statistically different for $p < 0.05$.

3. Results

3.1. Media formulation does not affect removal of mucus or epithelial cells

For the purpose of lamina propria leukocyte isolation, conditions resulting in the rapid removal of epithelial cells were considered optimal. Complete epithelial cell removal was identified by visual observation of solution clarification as described in Section 2.5.1. The effects of DTT and EDTA concentrations on removal of mucus and epithelial cells were investigated utilizing BALB/c and ICR mice ($n = 19$ total). These studies demonstrated that regardless of the DTT (2 or 5 mM) or EDTA (5 or 30 mM) concentration used, epithelial cells were removed in a similar number of washes, and neither DTT nor EDTA concentration had an effect on cell viability (Table 2). Therefore 5 mM DTT was chosen as there was a trend towards increased viability as compared to 2 mM DTT. 5 mM EDTA was selected over 30 mM EDTA because 30 mM EDTA provided only minor increases in epithelial cell removal rates.

3.2. Wash temperature has a significant effect on mucus and epithelial cell removal

Utilizing optimized DTT and EDTA concentrations, we next investigated the effect of temperature. Tissues from

Table 2

Effect of media composition, incubation temperature, wash frequency and time on epithelial cell removal.

Media composition				
Wash solutions				
DTT	EDTA	Supplement	% Viability	Total washes ^a
2 mM	5 mM		52.67	10
2 mM	30 mM		47.88	9
5 mM	5 mM		59.03	10
5 mM	30 mM		57.05	9
5 mM	5 mM	2% FBS	56.57	10
5 mM	5 mM	10 mM HEPES	51.93	10
5 mM	5 mM	20 mM HEPES	52.1	10
Temperature				
Wash temperature		% Viability	Total washes ^a	
4 °C		44.51 \pm 2.56	12	
Room temperature		52.65 \pm 1.35 ^b	8	
37 °C		57.42 \pm 1.21 ^{c, d}	6	
Wash frequency and time				
Optimal conditions ^e				
Solution	Total washes ^a	Total time	Wash frequency	
5 mM DTT	2	20 min	1 \times 20 min	
5 mM EDTA	4	40 min	3 \times 15 min	
Normalization to tissue weight ^f				
Solution	Wash frequency	Volume per wash	Time per wash ^g	
5 mM DTT	1	20 ml/g	20 min/g	
5 mM EDTA	3	15 ml/g	15 min/g	

^a Ten min washes.

^b $p < 0.05$ compared to 4 °C.

^c $p < 0.001$ compared to 4 °C.

^d $p < 0.05$ compared to room temperature.

^e Determined in inbred 129SvEvTac mice.

^f Determined in outbred ICR mice.

^g Based on mean weight of all tissues within an experiment.

129S6/SvEvTac ($n = 4$ to 21) mice were washed in DTT and EDTA solutions at 4 °C, RT or 37 °C. In contrast to media formulation, incubation temperature had a significant effect on both removal rate and cell viability (Table 2). The number of washes required decreased with increasing temperature. Furthermore, cell viability was significantly increased at 37 °C as compared to RT ($p < 0.05$) and 4 °C ($p < 0.001$), and 37 °C as compared to RT ($p < 0.05$). Therefore incubation at 37 °C was found to be critical for efficient mucus and epithelial cell removal.

3.3. Mucus and epithelial cell removal can be adapted based on tissue weight

Optimal media formulation and incubation temperature conditions were used to determine optimal wash duration and frequency. 129S6/SvEvTac mice ($n = 2$ –6 per group) were used to identify that 20 min in DTT (two 10 min washes), and 40 min in EDTA (four 10 min washes) were required for mucus and epithelial cell removal,

respectively. Subsequently a single 20 min wash in solution 1 and three 15 min washes in solution 2 were identified as optimal for mucus and epithelial removal, respectively (Table 2). However, when these conditions were applied to tissues from outbred strains such as ICR mice, epithelial cell removal was incomplete. This finding prompted us to evaluate whether the protocol could be adapted based on total small intestinal (SI) tissue weight. One of the most striking observations when specific volumes were used was that an increased amount of tissue led to a greater pH change in wash solutions. Utilizing volumes and tissue weights from 129S6/SvEvTac mice, we determined that 20 ml solution 1 and 45 ml solution 2 were needed per gram of SI tissue (Table 2). After adjusting the volumes according to tissue weight, the pH of all samples remained consistent as determined by visual observation of phenol red in wash solutions. Despite increases in the volume of each solution, we were still unable to remove all epithelial cells when utilizing optimized times for the inbred mouse strains.

Experiments with media formulation had demonstrated that increasing EDTA concentration had little effect on the rate of epithelial cell removal (Table 2). Therefore, we investigated if increased incubation times were required for samples that required larger volumes. With optimized incubation times in 129S6/SvEvTac mice, we next determined that 20 min/g of tissue was required for DTT incubations and 45 min/g of tissue was required for EDTA solutions (~1 min per ml). However, while adjusting incubation time resulted in more consistent epithelial cell removal, sample to sample variability was still a problem. Therefore, we investigated if replenishing solutions by increasing the number of washes would improve consistency utilizing the average tissue weight from all samples within the experiment to calculate incubation times. Tissue weights from ICR mice ($n = 6$ – 10 per group) were used to adjust solution volume and time, and the total time was then divided into one, two, three or four incubations. We found that increasing the number of washes did indeed act as a buffer for eliminating sample variability, and that utilizing the mean tissue weight was an effective method for calculating incubation time. These studies revealed that one 20 min/g wash in solution 1 and three 15 min/g washes in solution 2 were required to consistently remove mucus and epithelial cells, respectively.

3.4. Additional experimental observations

Many of the results from optimization experiments were strictly observational and difficult to quantify, or were performed as preliminary studies. Given the important relevance of these observations, they were summarized below to further guide future investigations.

Initial experiments were performed to determine if treatment with DTT to remove mucus prior to epithelial cell removal was beneficial. These studies demonstrated that the same number of washes was required to remove all epithelial cells if only EDTA washes were performed, or if DTT and EDTA were combined in the same solution. Separate DTT and EDTA washes resulted in the ability to isolate an epithelial cell population with greater purity in a smaller volume. Therefore, DTT and EDTA washes were performed separately

to remove mucus and epithelial cells, respectively. Additional media supplementation for epithelial cell removal such as FBS or HEPES buffer did not significantly affect removal rates or cell viability. FBS was found to result in a slight increase in viability, while inclusion of HEPES buffer reduced removal rates. Therefore 2% FBS was included while HEPES was not used.

Finally, in addition to wash temperature we also tested optimal conditions for storage of samples between washes, as prolonged intervals between washes can occur when processing multiple samples. These studies demonstrated that, in contrast to incubation temperatures, storage at RT or on ice was significantly better than storage at 37 °C. Storage on ice was slightly better than storage at RT but this difference was not significant, and therefore in the current study, samples were stored at RT between washes.

3.5. Collagenase formulations differentially cleave surface markers

To the best of our knowledge the optimal collagenase formulation for global analysis of SI immune populations has not been reported. We next examined the effects of a standard amount of collagenase combined with either dispase (D) or thermolysin (T), at low (DL, TL), medium (TM), or high (DH, TH) concentrations within Liberase formulations commercially available from Roche. Tissues from BALB/c ($n = 3$) or ICR mice ($n = 2$) were processed according to the optimal mucus and epithelial cell removal protocols and tissues were then minced and digested at 0.15 Wünsch units/ml with one of five Liberase formulations (DL, DH, TL, TM or TH) and 200 Kunitz/ml DNase as described in Section 2.6.1. Analysis of MFI and percent of total cells on a marker specific level revealed a strong effect on lymphocytes with B-cell markers being cleaved with more aggressive formulations such as DH, TM and TH, and similar trends were observed for CD8 α . In contrast, the aggressive formulations such as TM and TH were significantly improved for CD3 and phagocytic markers (Fig. 1, Supplemental Fig. 1). These marker specific differences may be important for specific experimental goals. To quantitate Liberase effects on a global scale a scoring system was developed. For each marker, the Liberase formulation that maintained the highest level of marker integrity (strongest), and the formulation which resulted in the most marker degradation (weakest) was determined. Next the strongest/weakest frequency amongst the 20 markers was determined for each formulation and expressed as a ratio. This analysis revealed that Liberase TM was optimal for BALB/c mice and for percent of total cells in ICR mice, though Liberase DH was optimal for MFI in ICR mice (Table 3). Additional B cell markers were also investigated as Liberase TM appeared to adversely affect B cell markers. Both CD19 and Ly-6K were investigated for identification of B-cells and plasma cells, respectively. Unfortunately, both of these markers were also cleaved by the same formulations (DH, TM and TH) suggesting that B-cell markers are more susceptible to enzymatic cleavage (data not shown).

Marker cleavage is an important factor when choosing a collagenase formulation, and was considered in concert with other factors such as cost and cell viability. Amongst the Liberase formulations the TL formulation is presently the least expensive while the DL, DH, TM and TL formulations are

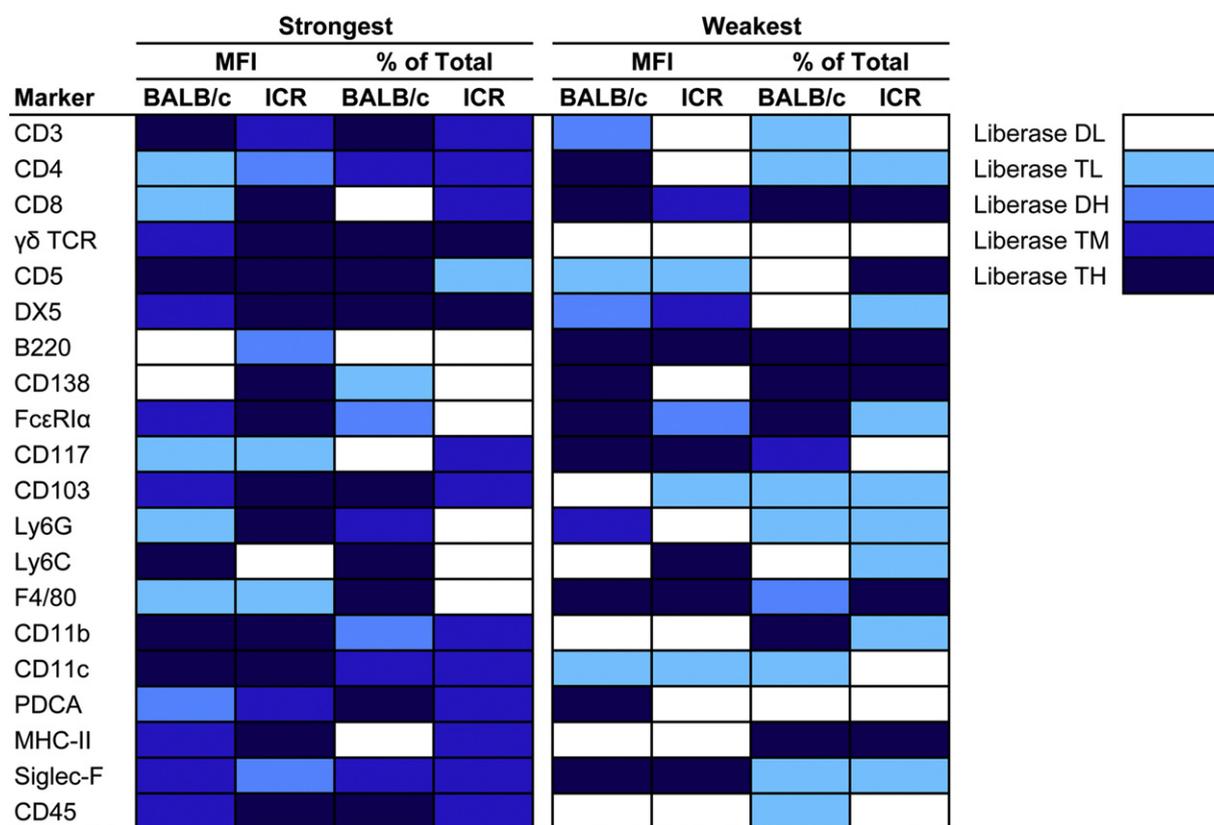


Fig. 1. Differential effects of collagenase formulations on marker expression. BALB/c ($n = 3$ per group) or ICR ($n = 2$ per group) mice were euthanized and small intestine tissue was processed according to the optimized epithelial removal steps (Table 2). Lamina propria tissue was then digested with one of five Liberase formulations at 0.15 units/ml, and stained for flow cytometry as described in Section 2.9. Mean fluorescent intensity (MFI) of marker positive cells or percentage of total cell values were determined. The collagenase formulation which resulted in the highest MFI or percentage of total cells (strongest) and the formulation which resulted in the lowest MFI or percentage of total cells (weakest) are shown. Results were then color coded according to the legend in the figure, and are presented as a heat map. Data are from two independent experiments.

2.6, 4.8, 2.2 and 4.0 times more costly, respectively. Based on marker cleavage and cost, Liberase TL and TM were investigated further. In an attempt to improve cell viability, 0.4 Wunsch units/ml TL and 0.2 Wunsch units/ml TM were compared based on the 2.2 \times increased cost of TM. Even with digestion at 0.4 Wunsch units/ml Liberase TL digestion with Liberase TM resulted in increased viability ($p = 0.04$, data not shown). Furthermore, increasing the TL concentration resulted in increased marker cleavage to a level nearly

equivalent to TM at 0.2 Wunsch units/ml (data not shown). Any further increase in Liberase TL concentration would not be cost effective, thus Liberase TM at 0.2 Wunsch units/ml was chosen for digestion.

Similar to mucus and epithelial cell removal procedures, we found that the efficacy of collagenase digestion varied based on tissue weight. 7 ml digestion solution per gram of SI tissue was found to be optimal. While incubation for 30 min did not affect viability of samples <1 g; samples >1 g required additional incubation time. For samples weighing >1 g the incubation time was adjusted to 30 min/g utilizing average the tissue weight within the experiment.

Table 3
Identification of optimal collagenase formulation for global analysis.^a

Liberase	MFI		% of total cells	
	BALB-c	ICR	BALB-c	ICR
DL	2/6 (0.3)	1/9 (0.1)	4/5 (0.8)	6/6 (1.0)
DH	1/2 (0.5)	3/1 (3.0) ^b	2/1 (2.0)	0/0 (0)
TL	5/2 (2.5)	2/3 (0.7)	1/7 (0.1)	1/8 (0.1)
TM	7/1 (7.0) ^b	2/2 (1.0)	4/1 (4.0) ^b	11/0 (NA) ^b
TH	5/9 (0.7)	12/5 (2.5)	9/6 (1.5)	2/6 (0.3)

^a For each formulation, a ratio indicates the frequency of strongest to weakest signal intensities from all 20 markers analyzed (Fig. 1). Analysis was performed on MFI and percent of total cells for both BALB/c and ICR mice.

^b Optimal formulation.

3.6. Density gradient centrifugation is not necessary for SI global immune phenotype analysis

Nearly every intestinal isolation procedure includes a density gradient purification step. However, we have found the density gradient step to be time consuming and that re-isolation of target cell layers was highly subjective. We next investigated the necessity of this step for digestion aimed at global analysis. Flow cytometry was performed on the same sample both pre- and post-density gradient centrifugation. An increase in total CD45⁺ leukocytes was observed following density gradient

centrifugation. However, when distributed across multiple populations this difference was diluted out and only CD4⁺ T cells were significantly increased, while NK cell and NK-T cell populations were significantly decreased (Fig. 2). Therefore, when balanced against additional time requirements and subjective nature of re-purification, the minor changes in population frequency suggested this step might not be necessary when isolation of a certain cell type is not required.

Next, we investigated the role of density gradient centrifugation in the removal of dead cells and debris, via trypan blue cell counting and flow cytometry. Following density gradient centrifugation an increase in viability was observed ($p < 0.001$), although a trend towards a reduction in total viable cells was also observed (Fig. 2). Optimization of the digestion procedure with Liberase TM resulted in significantly increased viability and total cell number over post-density gradient centrifugation samples ($p < 0.001$) (Fig. 2). To investigate debris removal, live/dead fluorescent staining was utilized to identify live cells, dead cells and debris as described in Section 2.8. This analysis revealed a significant correlation between pre-density viability and debris removal as samples with lower pre-density viabilities had more debris removed by density gradient centrifugation ($p < 0.01$, data not shown). Interestingly, in samples with viability $\leq 80\%$, more debris was observed following density gradient centrifugation, suggesting the need for density gradient centrifugation can be prevented by optimizing collagenase digestion procedures.

3.7. Variation across isolation methods can significantly affect interpretation of results

The relevance of SI tissue processing methods was compared via results obtained with the previous and optimized protocols performed in our laboratory. In these experiments 129S6/SvEvTac mice ($n = 10$ – 18) were euthanized and lamina propria leukocytes were isolated from intestinal tissue processed according to the previous isolation procedure (Sections 2.5.2 and 2.6.2) or according to the optimized procedure described in the current manuscript (Sections 2.5.1 and 2.6.1). This comparative protocol analysis revealed significantly different outcomes for both lymphocytes and phagocytic cells (Fig. 3). Despite elimination of the density gradient purification step, the majority of differences were observed as increases in specific populations on a percentage basis. We detected decreases in CD8⁺ T cells, B cells and plasma cells, and these differences were likely due to marker cleavage by Liberase TM (Fig. 2). These results highlight the difficulties associated with comparing results between experiments that utilized different isolation procedures, and the need to standardize the murine intestinal leukocyte isolation procedure.

3.8. Intraepithelial and lamina propria populations isolated with the optimized protocol

While the main goal of the current protocol was to isolate lamina propria cells, we also collected epithelial cells for flow cytometric analysis. Normal populations in 129S6/SvEvTac mice were identified as our laboratory utilizes these mice for *Salmonella* infection and dietary interventions (Henderson et

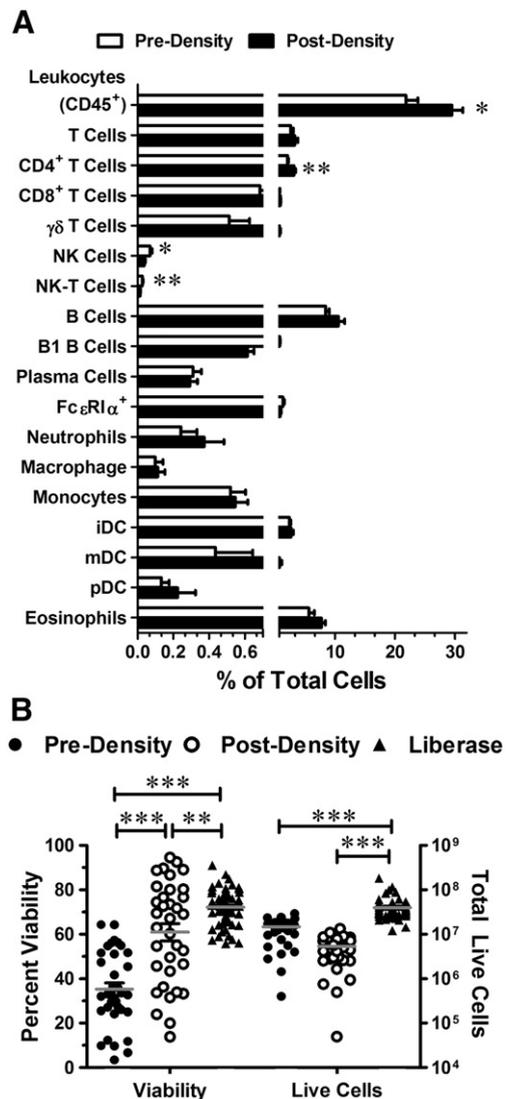


Fig. 2. Density gradient centrifugation is not necessary for global immunophenotyping. (A) 129S6/SvEvTac mice ($n = 9$) were euthanized and small intestine tissue was digested with collagenase D and analyzed by flow cytometry before and after density gradient centrifugation as described in Sections 2.7 and 2.9. Data were pooled from 2 independent experiments and statistical differences were determined by a two-tailed Student's *t*-test. (B) Percent viability and total live cells and were determined by trypan blue cell counts. For pre- and post-digestion groups samples were digested with collagenase D and data were pooled from six experiments ($n = 35$). Samples from BALB/c ($n = 15$), 129S6/SvEvTac ($n = 12$) and ICR ($n = 8$) mice were analyzed both before and after density gradient centrifugation. For the Liberase group 65 samples from BALB/c ($n = 5$) 129S6/SvEvTac ($n = 42$), and ICR ($n = 16$) mice were digestion with 0.2 units/ml Liberase TM. Individual values are graphed and gray bars indicate the mean value. Percent viability is graphed on the left axis and total viable cells on the right axis. Statistical differences were determined by a one-way ANOVA followed by a Tukey's multiple mean test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

al., 2012; Kumar et al., 2012). 129S6/SvEvTac mice ($n = 9$) were processed according to the optimized protocol described in Sections 2.5.1 and 2.6.1. Similar frequencies of total T cells were observed in the epithelium and lamina propria, though CD8⁺ T cells were more prominent in the epithelium,

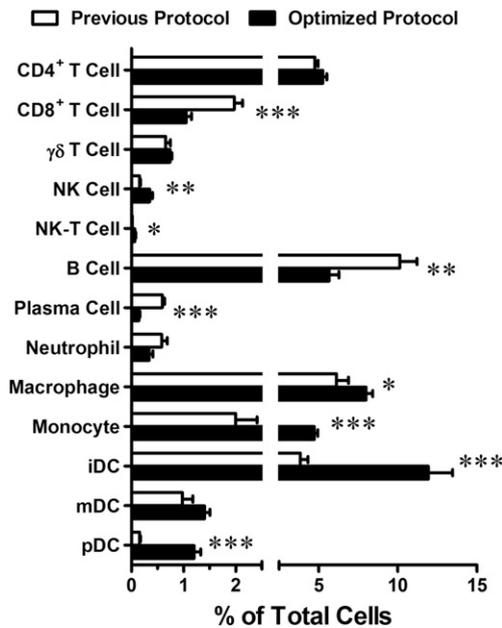


Fig. 3. Variation in isolation procedure results in significant changes in cell populations. 129S6/SvEvTac mice ($n = 10$ per group) were euthanized and processed according to the collagenase D protocol followed by density gradient centrifugation (previous protocol), or were processed according to the Liberase TM protocol (optimized protocol). Data were pooled from two independent experiments. Statistical differences between previous and optimized protocols were determined by a Student's two tailed t -test. Alternative population definitions were used for some populations including: CD4⁺ T cells: FSC-SSC^{low}/DX5⁻/CD4⁺; CD8⁺ T cells: FSC-SSC^{low}/DX5⁻/CD8^α⁺; NK Cells: FSC-SSC^{low}/CD4⁻/CD8⁻/DX5⁺; NK-T cells: FSC-SSC^{low}/DX5⁺/CD4⁺ or CD8^α⁺; Macrophages: CD45⁺/CD11b⁺/Ly6G⁻/Ly6C⁻/F480⁺; CD103 DCs: CD45⁺/CD11c⁺/CD103⁺; myeloid DCs: CD45⁺/CD11c⁺/CD101b⁺; PDCs: CD45⁺/CD11c⁺/PDCA⁺. Total T cells, B1 B cells, total allergy (FcεR1α⁺), basophils, mast cells and eosinophils were not investigated.

while CD4⁺ T cells were more prominent in the lamina propria (Fig. 4). Finally, the optimized protocol developed in this study is summarized in a flow chart highlighting the conditions identified to be important for efficient epithelial cell removal and lamina propria cell isolation (Fig. 5).

4. Discussion

This report describes optimization of an intestinal isolation procedure that can be standardized according to tissue weight and that demonstrated a reproducible, comprehensive intestinal immune phenotype analysis. Incubation temperature was determined to be a critical variable for mucus and epithelial cell removal, while media formulation played a minor role. The utilization of tissue weight to correct solution volume and incubation time, as well as optimization of wash frequency were the factors required for consistent removal of intestinal epithelial cells. Collagenase digestion was improved to maintain marker integrity and maximize cell viability in a cost effective manner. The optimized protocol was effective for isolation of intestinal cells from both inbred and outbred mouse strains.

Strain specific differences were also identified. For example, the mucus and epithelial cell removal steps optimized for 129 SvEvTac mice were not as effective in ICR mice. Furthermore, strain specific differences in enzymatic marker cleavage including Ly6C and Ly6G expression, as well as CD8 were observed (Fig. 1, Supplementary Fig. 1). These differences highlight difficulties in broadly applying protocols that were optimized with specific mouse strains. In the current study we identified tissue weight as an effective method for normalization of mucus and epithelial cell removal across mouse strains. Differences in the susceptibility of specific markers to collagenase cleavage remain a concern. However, identification of Liberase TM as the optimal formulation for global analysis in two mouse strains suggests this formulation may be optimal across strains.

Improvements in mucus and epithelial cell removal as well as identification of optimal digestion techniques for global analysis were also validated by total cell yields and observed immune cell populations. For example, the current procedure yielded an average of 1.3×10^7 viable lymphocytes based on cell counts and CD45 staining (Fig. 2, data not shown). This is in agreement with previous reports of 1×10^7 lymphocytes or $2\text{--}5 \times 10^6$ lymphocytes per intestine (Davies and Parrott, 1981; Sheridan and Lefrancois, 2012). The average viability of 72.1% obtained in this study was similar to the 50–75% reported for samples prior to density gradient centrifugation (Lyscom and Brueton, 1982). Although the epithelial cell isolation was not the focus of this study, viability results were similar to that previously reported, $48 \pm 17\%$ (Leventon et al., 1983).

With regard to immune populations we observed large numbers of CD8⁺ and γδ T cells in the epithelium with higher levels of CD4⁺ T cells in the lamina propria as described previously (De Geus et al., 1990; Gautreaux et al., 1994; Ye et al., 2010). Furthermore B cells were essentially absent from the epithelial fraction (Suzuki, 2012). Also, the majority of iDCs were localized within the lamina propria (Farache et al., 2013). Unfortunately, comparison of specific frequencies between studies proved difficult because previous studies reported percentages of total lymphocytes rather than total intestinal cells.

Such difficulties comparing results between gut immune cell phenotype studies that utilize different isolation protocols has been a topic of discussion for some time (Van der Heijden and Stok, 1987; Kearsley and Stadnyk, 1996). Indeed, the current study demonstrates that significant differences can be obtained simply by altering the isolation protocol (Fig. 4). The current protocol provides an attractive option for standardization as multiple variables were optimized based on cell viability. Furthermore, this report provides a template for implementation of minor modifications required for specific experimental needs. For instance, in our own studies investigating dietary interventions we have observed certain diets increase mucus production resulting in incomplete epithelial cell removal. With the knowledge that increasing the number of washes can buffer out such slight variances we were able to perform two 10 min DTT washes rather than one 20 min wash and subsequently removed all epithelial cells without changing the epithelial wash procedure. By identifying the relevance of certain conditions to the effective isolation of epithelial and lamina propria cells, small changes in the protocol can be made to minimize variation between experiments, resulting in more comparable results.

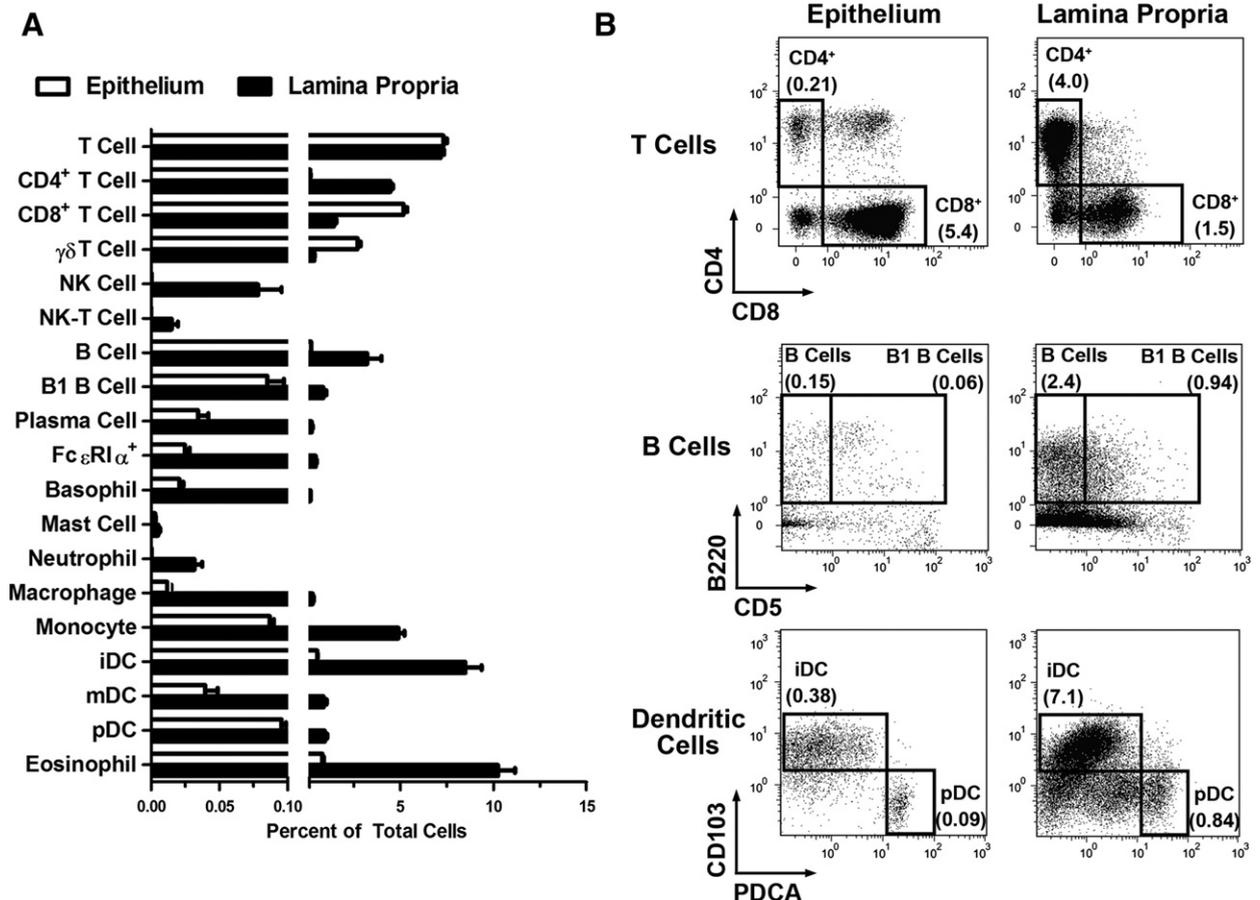


Fig. 4. Intraepithelial and lamina propria leukocyte populations. (A). 129S6/SvEvTac mice ($n = 9$) were euthanized and processed according to the Liberase digestion protocol. Single cell suspensions were stained and analyzed by flow cytometry as described in Section 2.9. Data were pooled from two independent experiments. (B). Representative dot plots from epithelium and lamina propria cell populations isolated from 129S6/SvEvTac mice. Preliminary gating strategies for representative dot plots include: T cells: FSC-SSC^{low}/DX5⁻/CD3⁺; B cells: FSC-SSC^{low}/CD45⁺/CD3⁻/DX5⁻; Dendritic cells: CD45⁺/Siglec-F⁻/CD11c⁺/MHC-II⁺.

Optimization of the collagenase digestion for global phenotyping was one of the major improvements in the current report. However, B cell markers were disproportionately affected (Fig. 3). As increasing TL formulations to concentrations which result in acceptable cell viability was found to cleave B cell markers, mechanical disruption followed by density gradient centrifugation may be a more effective technique for B cell investigations. Specific B cell isolation procedures have also been reported, although the effects of the crude collagenase used in these studies on marker expression were not investigated (Cebra et al., 1977; Lycke, 1986; Van der Heijden and Stok, 1987). Purified collagenase formulations from Worthington, Sigma or other manufacturers may also be more effective for B cell isolation, although this will need to be tested and weighed against the effects on other immune cell markers.

Finally, removal of the density gradient centrifugation steps was one of the major time saving modifications allowing a higher number of mouse intestinal samples to be processed at one time. In agreement with previous studies we observed that while dead cells and debris were removed, the total number of viable cells was also reduced (Lyscom and Brueton, 1982; Van

der Heijden and Stok, 1987). However, at viabilities of $\geq 80\%$ this effect appears to be lost. Due to increased time requirements and the subjective nature of density gradient centrifugation, this step was eliminated from the current protocol. Improving the collagenase digestion step enhanced cell viability, though dead cells were still present. To ensure analysis of live cells by flow cytometry, newly available fixable viability dyes could be used.

In summary, this report provides an optimized protocol for isolation of lamina propria leukocytes for global immune phenotype analysis. The flexibility and identification of key variables affecting isolation will allow for rapid application based on experimental needs. These results provide rationale for further studies to more precisely optimize epithelial cell isolation conditions. Given that similar techniques have been reported for stomach, cecum and colon (Weigmann et al., 2007; Lee et al., 2009; Ruiz et al., 2012), this optimized procedure may also be applicable, and potentially reduce the time needed for isolation of the entire gastrointestinal mucosa. Finally, results from the current investigation may also be applicable or adaptable to tissues from other species including humans.

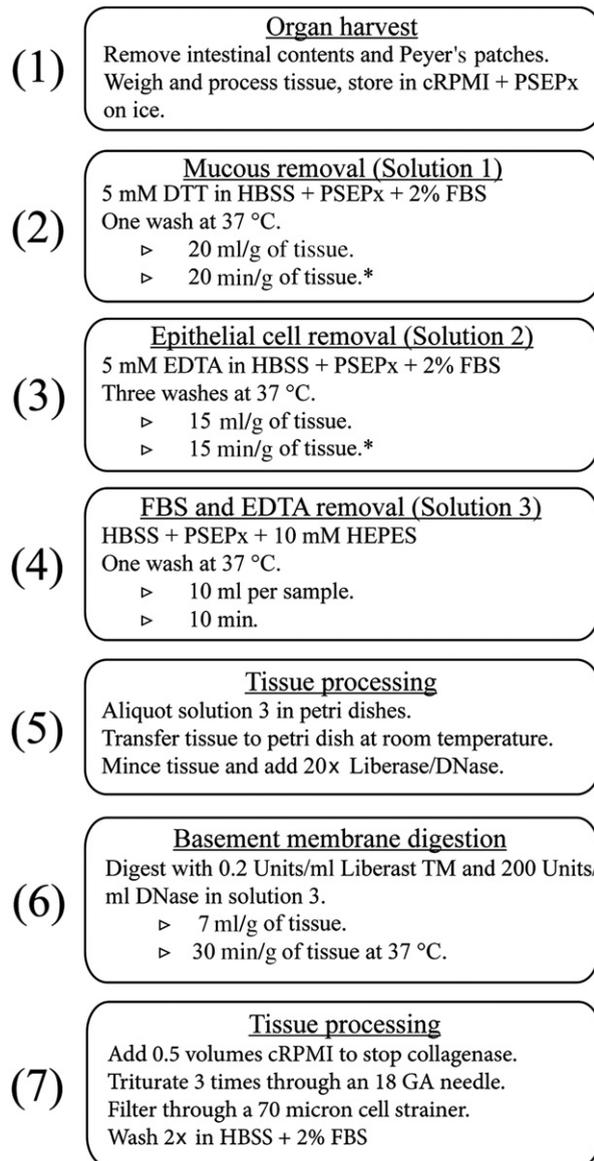


Fig. 5. Flow diagram of optimized intestinal digestion procedure. * = Incubation time is calculated from the mean tissue weight for animals within the experiment.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jim.2014.01.014>.

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