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Efficient Isolation of Lamina Propria Mononuclear Cells from Murine Colon Using Collagenase E

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TITLE:
Isolation of Lamina Propria Mononuclear Cells from Murine Colon using Collagenase E

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KEYWORDS:
Lymphocytes; colon; murine; lamina propria; colitis; transplantation; flow cytometry; enzymatic digestion; inflammation; mononuclear cells

SUMMARY:
The goal of this protocol is to isolate mononuclear cells that reside in the lamina propria of the colon by enzymatic digestion of the tissue using collagenase. This protocol allows for the efficient isolation of mononuclear cells resulting in a single cell suspension which in turn can be used for robust immunophenotyping.

ABSTRACT:
The intestine is the home to the largest number of immune cells in the body. The small and large intestinal immune systems police exposure to exogenous antigens and modulate responses to potent microbially derived immune stimuli. For this reason, the intestine is a major target site of immune dysregulation and inflammation in many diseases including but, not limited to inflammatory bowel diseases such as Crohn's disease and ulcerative colitis, graft-versus-host disease (GVHD) after bone marrow transplantation (BMT), and many allergic and infectious conditions. Murine models of gastrointestinal inflammation and colitis are heavily

used to study GI complications and to pre-clinically optimize strategies for prevention and treatment. Data gleaned from these models via isolation and phenotypic analysis of immune cells from the intestine is critical to further immune understanding that can be applied to ameliorate gastrointestinal and systemic inflammatory disorders. This report describes a highly effective protocol for the isolation of mononuclear cells (MNC) from the colon using a mixed silica-based density gradient interface. This method reproducibly isolates a significant number of viable leukocytes while minimizing contaminating debris, allowing subsequent immune phenotyping by flow cytometry or other methods.

INTRODUCTION:

Though the gastrointestinal (GI) tract is primarily dedicated to the processing and reabsorption of nutrients from food, the GI tract also maintains central roles in the integrity of the vascular, lymphatic, and nervous systems and of numerous other organs through its mucosal and submucosal immune system[1]. The GI immune system has an influential role in both gastrointestinal and systemic health due to its constant exposure to foreign antigens from food, commensal bacteria, or invading pathogens[1,2]. Thus, the GI immune system must maintain a delicate balance in which it tolerates non-pathogenic antigens while responding appropriately to pathogenic antigens[1,2]. When the balance of tolerance and defense is disrupted, localized or systemic immune dysregulation and inflammation can occur resulting in a myriad of diseases[1-3].

The intestine harbors at least 70% of all lymphoid cells in the body[4]. Most primary immunologic interactions involve at least one of three immune stations in the intestine: 1) Peyer's Patches, 2) Intraepithelial lymphocytes (IEL) and 3) lamina propria lymphocytes (LPL). Each of these is comprised of a complex interconnected network of immune cells that rapidly respond to normal immune challenges in the gut[5]. Restricted to the stroma above the muscularis mucosae, the loosely structured lamina propria is the connective tissue of the gut mucosa and includes scaffolding for the villus, the vasculature, lymphatic drainage, and mucosal nervous system, as well as many innate and adaptive immune subsets[6-9]. LPL are comprised of $CD4^+$ and $CD8^+$ T cells in an approximate ratio of 2:1, plasma cells and myeloid lineage cells including, dendritic cells, mast cells, eosinophils and macrophages[6].

There is a growing interest in understanding the immune dysregulation and inflammation of the gut as it pertains to various disease states. Such conditions as Crohn's disease and ulcerative colitis all manifest varying levels of colonic inflammation[10-12]. Additionally, patients with malignant or non-malignant disorders of the marrow or immune system who undergo an allogeneic bone marrow transplantation (allo-BMT) can develop various forms of colitis including 1) direct toxicity from conditioning regimens before BMT, 2) infections caused by immunosuppression after BMT and 3) graft-versus-host disease (GVHD) driven by donor-type T cells reacting to donor allo-antigens in the tissues after BMT[13-15]. All these post-BMT complications result in significant alterations in the immune milieu of the intestines[16-18]. The proposed method allows a dependable assessment of immune cell accumulation in the mouse colon and, when applied to murine recipients after BMT, facilitates an efficient assay of both donor and recipient immune cells involved in transplant tolerance[19,20]. Additional causes of gut inflammation include malignancies, food allergies, or disruption of the gut microbiome. This

protocol allows access of gut mononuclear cells from the colon and, with modifications, to leukocytes of the small intestine in any of these preclinical murine models.

A PubMed search using the search terms “intestine AND immune cell AND isolation” reveals over 200 publications describing methods for small intestine digestion to extract immune cells. However, a similar literature search for colon yields no well-delineated protocols specifying isolation of immune cells from the colon. This may be because the colon has more muscular and interstitial layers, rendering it more difficult to completely digest than the small intestine. Unlike existing protocols, this protocol specifically uses Collagenase E from *Clostridium histolyticum* without other bacterial collagenases (Collagenase D/ Collagenase I). We demonstrate that, using this protocol, digestion of the colonic tissue can be achieved while preserving the quality of isolated gut mononuclear immune cells (MNC) without the addition of anti-clumping reagents such as sodium versenate (EDTA), Dispase II, and deoxyribonuclease I (DNAse I)[21-23]. This protocol is optimized to allow reproducible robust extraction of viable MNC from the murine colon for further directed studies and should lend itself to the study of immunology of the colon or (with modifications) the small intestine[24,25].

PROTOCOL:

All studies were conducted under rodent research protocols reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Miami Miller School of Medicine, which meets the veterinary standards set by the American Association for Laboratory Animal Science (AALAS).

1. Preparation of solutions

1.1. As described in **Table 1**, prepare the Colon Buffer, Silica-Based Density Separation Media 100%, Silica-Based Density Separation Media 66%, Silica-Based Density Separation Media 44%, Collagenase E Digestion Buffer, and FACS Buffer.

1.1.1. Prepare Colon Buffer the day prior to the procedure and store overnight at 4 ˚C.

1.1.2. Prepare 100% Silica-Based Density Separation Media the day prior to the procedure and stored overnight at 4 ˚C, placing at room temperature the morning of the procedure to thaw.

1.1.3. Prepare 66% and 44% Silica-Based Separation Media in the morning of the isolation, using room temperature 100% Silica-Based Density Separation Media and Colon Buffer.

1.1.4. Measure the appropriate amount of *Clostridium histolyticum*-derived Collagenase E and store at -20 ˚C overnight prior to the procedure. The following morning, dissolve in the appropriate volume of Colon Buffer to derive Collagenase Digestion Buffer. For all incubations with Collagenase Digestion Buffer on day of the procedure, pre-warm the solutions to 37 ˚C.

1.2. For the entire of the protocol steps, keep one centrifuge at 20 ˚C and the rotation speed of 859 x *g*, with brakes inactivated (0 deceleration) for gradient centrifugation. Set another to 4 ˚C

and rotation speed of 859 x *g*, with the standard deceleration, for wash steps.

2. Harvesting the Colon

2.1 Euthanize the mouse via CO_2 asphyxiation followed by AALAC-approved confirmatory method.

2.2 Place the mouse in a supine position and spray the fur with 70% ethanol. Using large tissue scissors, make a vertical midline incision and expose the intact peritoneum.

2.3 Using fine dissection scissors, open the peritoneum. Use forceps to move the small bowel to one side and expose the descending colon. Slightly pull upward on the descending colon to maximally expose the rectal portion of the colon. Cut the distal rectum deep in the pelvis and dissect and remove the entire colon as one unit, from the distal rectum to cecal cap.

2.4 Transfer the colon in 20 mL chilled Colon Buffer in a 50-mL polypropylene tube.

3. Cleaning the Colon

3.1 Place the colon on a moistened paper towel and extract the solid stool by applying mild pressure to the bowel wall with the blunt end of scissors.

3.2 Place the colon in a Petri dish and flush the gut with 10 mL of chilled Colon Buffer using a 10 mL syringe with 18 G blunt fill needle.

3.3 Transfer the colon to a Colon Buffer moistened paper towel and remove the mesentery and fat with the sharp end of scissors.

3.4 Place the colon in a Petri dish filled with 5-10 mL chilled Colon Buffer agitating manually to wash remaining colonic contents. Repeat 2-3 times.

3.5 Cut the colon longitudinally from its more muscular rectal end to the proximal colon (generating a single rectangular open colon piece) in a Petri dish filled with fresh chilled Colon Buffer. Discard existing media and refill with clean chilled Colon Buffer.

3.6 Wash the intestine 3 times by vigorously swirling it in the Petri dish and replacing the 5-10 mL of chilled Colon Buffer after with each wash.

3.7 Place the rectangular colon tissue on a paper towel moistened with Colon Buffer and cut it by slicing it horizontally and then into small fragments (3 mm x 3 mm sections).

3.8 Collect the colon fragments carefully using fine forceps into 20 mL chilled Colon Buffer in a 50 mL polypropylene conical tube.

3.9 Wash the colon fragments 3 times, each wash in 20 mL Colon Buffer, by vigorously swirling the tube for 30 s. Between each agitation, allow the tissue fragments to settle to the bottom of the tube. Decant or vacuum aspirate the supernatant while preventing tissue fragment loss in the aspiration process between each wash.

NOTE: There is no need to change the tube after each wash.

4. Collagenase Digestion 1

4.1 Add 20 mL of the Collagenase Digestion Buffer to the washed colon fragments in the 50 mL polypropylene conical tube.

4.2 Place the closed 50 mL tube at 37 ˚C in an incubated orbital shaker with the rotation rate set at 2 x *g* for 60 min. Ensure the tissue fragments are in constant motion during agitation; if necessary, increase the rotation rate incrementally to ensure that no tissue fragments settle to the tube bottom.

5. Prepare Silica-Based Separation Media Gradients

5.1 Prepare 66% and 44% Silica-Based Density Separation Media, using 100% Silica-Based Density Separation Media at 20 ˚C (room temperature) and Colon Buffer.

5.2 Pour 5 mL of 66% Silica-Based Density Separation Media into each of 3 separate 15 mL polypropylene tubes. Prepare 3 tubes per colon. This forms the higher density base of the gradient isolation procedure, onto which lower density separation media will be layered to create the separation gradient.

5.3 Store at 20 ˚C until use.

6. Collection of Supernatant from Digestion 1

6.1 Collect only the supernatant using a 25 mL serological pipette and filter the supernatant through a 40 μm pore filtration fabric cell strainer placed into a clean 50 mL polypropylene conical tube, after Collagenase Digestion 1 is completed. Be careful not to aspirate any existing tissue fragments.

NOTE: Retain any remaining visible tissue fragments in the tube. These will undergo second collagenase digestion (step 8).

7. Quenching Collagenase Digestion Buffer

7.1 Fill the 50 mL polypropylene tube completely with chilled Colon Buffer.

NOTE: Collagenase is active at 37 ˚C; hence chilled buffer inactivates this enzyme.

7.2 Centrifuge the tube at 4 ˚C at 800 x *g* for 5 min.

7.2.1 Discard the supernatant via vacuum aspiration. Wash cells with 25 mL of fresh Colon Buffer and centrifuge at 800 x *g* for 5 min.

7.2.2 Resuspend the pellet in less than 1 mL of fresh chilled Colon Buffer.

7.2.3 Place the 50 mL polypropylene conical tube on ice.

8. Collagenase Digestion 2

8.1. Repeat step 4 (Digestion 1) with the remaining tissue fragments retained from step 6.1.

9. Tissue disaggregation following Digestion 2

9.1 Flush the tissue fragments vigorously back and forth between the tube and a 10 mL syringe through an 18-gauge blunt-end needle.

9.2 Repeat this flush for a minimum of 7-8 complete passages, continuing until no gross tissue fragments or debris are visible.

10. Filter Cells

10.1 Pass the tissue disaggregation suspension through a 40 µm-pore filtration fabric cell strainer into a clean 50 mL polypropylene tube.

10.2 Wash the filtration fabric cell strainer with 10 mL chilled Colon Buffer to recover any cells ensnared in the filter.

11. Quenching Collagenase Digestion

11.1 Fill the 50 mL polypropylene conical tube to the rim with chilled Colon Buffer.

NOTE: The temperature of Colon Buffer is critical to ensure quenching of collagenase activity.

11.2 Spin at 4 ˚C and 800 x *g* for 5 min.

11.3 Discard the supernatant via vacuum aspiration.

11.4 Wash by resuspending in 25 mL of fresh chilled Colon Buffer**,** followed by centrifugation at 4 ˚C, 800 x *g* for 5 min.

11.5 Discard the supernatant via vacuum aspiration.

11.6 Pool the resuspended pellet from Collagenase Digestion 1 (step 7) to its corresponding tube from step 11.4.

11.7 Repeat Step 11.4 (wash and centrifugation).

12. Silica-Based Density Separation Media Gradient Separation

NOTE: Perform steps 12-18 as quickly as possible, to ensure rapid quenching of collagenase activity.

12.1 Following step 11.7, resuspend each pellet in 24 mL total of 44% Silica-Based Density Separation Media per colon.

12.2 Slowly layer 8 mL of the media from step 12.1 onto each of three tubes prepared at step 5.2 (containing 66% Silica-Based Density Separation Media), using a 10 mL serological pipette. Maintain a steady and slow flow of the 44% Density Separation Media while layering the gradient in order to avoid disruption of the interface.

12.3 Carefully balance all tubes within the centrifuge buckets using a weigh scale.

12.4 Spin the tubes 20 min at 859 x *g* in a centrifuge without brake at 20 ˚C. Allow the rotors to come to complete rest before removing tubes, taking care not to disrupt the cells at the gradient interface.

13. Collect mononuclear cells from the gradient interface

13.1 Visualize the gradient interface (near the 5 mL mark), where typically a 1-2 mm thick white band (containing MNC) is present.

NOTE: One may or may not see a white band. However, MNC will be at this interface and should cloud the clarity of the gradient interface.

13.2 Vacuum aspirate and discard the top 6 mL of the top gradient to allow easier pipette access to the interface.

13.3 Using continuous manual suction and steady rotating wrist motion, collect the interface layer of cells into a clean 50 mL polypropylene conical tube. Collect until the interface between the 2 gradients is clear and refractile (clear of cells).

13.4 Fill the collection tube with 50 mL of chilled FACS Buffer. Spin at 4 ˚C, 800 x *g* for 5 min.

13.5 Aspirate the supernatant via vacuum aspiration and resuspend the pellet in 1 mL of FACS Buffer.

13.6 Count the cells on a hemocytometer at a 1:2 dilution using appropriate dead cell exclusion methods.

13.7 Proceed to FACS staining or other assays with freshly isolated colonic MNC.

REPRESENTATIVE RESULTS:

When working with murine colon disease models, it is helpful to be able to both quantify and qualitatively assess, among the MNC of the colon, multiple immune cell subsets involved in the inflammatory process. The single-cell suspension of MNC obtained through the application of this protocol facilitates such phenotypic characterization in a robust and reproducible manner. As a proof of principle for the application of this isolation method under diverse experimental settings, we retrieved colonic MNC using this method and performed multi-parameter flow cytometry on cells isolated from mice with **(Figures 1 and 2, allogeneic BMT**) and without **(Figure 2A, syngeneic BMT)** significant immune-mediated colonic injury following BMT.

Flow cytometry and data analyses were performed to compare the fractions of apoptotic and necrotic dead lymphocytes when using either Collagenase E or D for the isolation, with or without DNase 1 treatment. The gating strategy used during flow cytometry is provided in **Figure 1A**. Following Annexin V (apoptosis marker) and fixable Live/Dead Blue dye (necrosis marker) staining on single-cell suspensions following each isolation, Collagenase E without DNase showed a significantly higher percentage of Annexin V^{neg}Live/Dead $Blue^{neg}$ live cells (median 43.23%, n = 3) after isolation when compared to Collagenase D without DNase (median 7.49%, n = 3), even when compared to Collagenase E + DNase (median 11.1%, n = 3) or Collagenase D + DNase (median 15.47%, n = 3). In addition, we identified Annexin V^{neg}Live/Dead Blue+ necrotic cells at a median percentage of 45.6% in the Collagenase E group (n = 3) versus 85.1% in the Collagenase D group (n = 3), 73.7% in the Collagenase E + DNase group, and 85.07% in the Collagenase D + DNase group, respectively (n = 3). Representative FACS plots from n =1 animal in each group are shown **Figure 1B**).

As further proof of principle of the consistency and yield of viable MNC using this procedure in diseased mice, multi-parametric flow cytometry was applied to the MNC isolated from CD45.2 BALB/c recipient mice on day 7 after receiving BMT of either allogeneic (CD45.1 C57BL/6 donor) or syngeneic (CD45.1 BALB/c donor) BMT models. Using absolute MNC numbers multiplied by percentage gated immune subsets obtained by flow cytometry analyses, mean absolute numbers of donor $CD4^+$ and $CD8^+$ T cells extracted from the BMT recipient's colon could be calculated and compared (n = 4 per group, **Figure 2A**). Since it can be important to identify and/or quantitate rare immune cell populations in such mouse models, we assessed rare subsets including donor derived (CD45.1+) Foxp3+ T regulatory cells (Treg) in both syngeneic and allogeneic BMT models. The gating strategy to reach donor Treg cells ($CD4^+CD25^+FoxP3^+$) from the antibody-stained single cell suspension is shown *(*sequence of gates delineated by a red arrow**; Figure 2B)**. Using this method, even rare subsets such as donor derived colonic Treg infiltrating recipient mouse colon after BMT could be analyzed (**Figure 2C,** representative plot; n = 1).

Figure 3 shows an extended application of this method in historic data from our group using the presented protocol to compare accumulation of GVHD-inducing CD8+ versus CD4+ donor-derived T cells in the colon of BALB/c mice either protected or not protected from GVHD by the pre-BMT treatment preparative (conditioning) regimen[20]. The tested preparative regimens included 800 cGy/myeloablative total body irradiation (TBI800) or non-myeloablative TBI (400TBI), as well as nonmyeloablative conditioning using total lymphoid irradiation (TLI) in which irradiation was delivered to the lymph nodes, thymus, and spleen with shielding of the skull, lungs, limbs, pelvis and tail. All conditioning was combined with anti-thymocyte serum (ATS), an immunomodulating agent. As early as day 6 after BMT, this colonic MNC isolation protocol resulted in robust flow cytometric analyses as compared to identical analyses on more lymphocyte enriched GVHD target organs such as spleen and mesenteric lymph nodes (MLN) (**Figure 3A)**[20]. Reproducible isolation of colonic MNC across BMT recipients (n = 7-10 per treatment group) allowed for a robust statistical comparison of absolute numbers of donor $CD8^+$ effector T cells between different pre-transplant conditioning treatment groups **(Figure 3B)**, yielding important data on immune phenotypes that led to key studies revealing the innate immune mechanisms of GVHD protection from TLI as opposed to TBI pre-BMT conditioning.[20]

FIGURES AND TABLES:

[Insert Figure 1]

[Insert Figure 2]

[Insert Figure 3]

FIGURE LEGENDS AND TABLES:

Figure 1: Flow cytometric analysis of colonic MNC at Day 7 after BMT in allogeneic mouse model systems when isolated with Collagenase E and D with and without DNAse 1. Wild-type (WT) ($CD45.2^+$) BALB/c ($H2K^{d+}$) mice received BMT from CD45 congenic ($CD45.1^+$) C57BL/6 donor mice (allogeneic BMT, n = 3 per group). WT ($CD45.2^+$) BALB/c recipient mice were administered 800cGy TBI (BALB/c) 1 day before BMT. At day 7 after BMT, single-cell suspensions of recipient colon were prepared following the methods of this manuscript with the use of Collagenase E (100 U/mL), Collagenase E (100 U/mL) with DNAse 1 (500μg/mL), Collagenase D (500μg/mL), or Collagenase D (500μg/mL) with DNAse 1 (500μg/mL) (n = 3 per group). Cells were stained with Live/dead-UV450 (Live/Dead Blue), Annexin V-APC, H-$2K^d$-PE, CD45.1-BV605, CD3-FITC, CD4-BV711, CD8-APC-Cy7, FoxP3-Pacific Blue, and CD11b-PE-Cy7 antibodies. (**A**) Gating strategy for FACS analyses. *Gate 0*, forward scatter (FSC-A) and side scatter (SSC-A) on the single-cell suspension of MNC used to identify leukocytes; *Gate 1*, exclusion of non-single cells using SSC-A; *Gate 2*, exclusion of non-single cells using FSC-A; *Gate 3*, identification of Annexin V-positive (apoptotic) and fixable viability dye Live/Dead-$UV450^+$Annexin $V^{-negative}$ (necrotic) cell subsets. (**B**) Representative FACS plots of Annexin V and fixable viability dye staining of gated leukocytes among MNC for the 4 experimental groups. N =1 representative mouse per group in groups: Collagenase E (100 U/mL), Collagenase E (100

U/mL) + DNAse 1 (500 μg/mL), Collagenase D (500 μg/mL), and Collagenase D (500 μg/mL) + DNAse 1(500 μg/mL).

Figure 2: Flow cytometric characterization of colonic MNC at Day 7 after BMT in allogeneic and syngeneic mouse model systems. WT (CD45.2$^+$) C57BL/6 (H2K$^{d\text{-}neg}$) and BALB/c (H2K^{d+}) mice received BMT from CD45-congenic (CD45.1$^+$) C57BL/6 and BALB/c donor mice (syngeneic or allogeneic BMT, n = 4 per experimental group). C57BL/6 and BALB/c recipient mice received preparative conditioning regimens of 950cGy (C57BL/6) and 800cGy (BALB/c) myeloablative TBI, delivered one day before BMT. At day 7 after BMT, single-cell suspensions of recipient colonic MNC were prepared following the methods of this manuscript. Cells were stained with Live-dead-BV510, H-2K^d-PE, CD45.1-BV605, CD4-FITC, CD8-APC-Cy7, CD25-PacificBlue, FoxP3-AF647, and CD11b-PE-Cy7 antibodies, N = 4 mice per group. (**A**) Mean ± SEM absolute number (log 10) CD45.1$^+$ H-2k$^{d\text{-}neg}$ or CD45.1$^+$ H-2k^{d+} (donor-type, in each case) CD11b^{neg}CD4$^+$ and CD8$^+$ T cells isolated from recipient colon at day 7 after conditioning and CD45.1 C57BL/6 (donor) → CD45.2 BALB/c (recipient) BMT. N = 4 per group. (**B**) Gating strategy for FACS analyses. *Gate 0*, forward scatter (FSC-A) and side scatter (SSC-A) on the single-cell suspension of MNC used to identify leukocytes; *Gate 1*, exclusion of non-single cells using SSC-A; *Gate 2*, exclusion of non-single cells using FSC-A; *Gate 3*, live cell selection *Gate 4,* separation of hematopoietic cells of BMT donor versus BMT recipient origin; *Gate 5,* selection of donor non-myeloid lineage cells; *Gate 6*, selective gating of CD4$^+$ T cells; *Gate 7,* separate gating of CD4$^+$CD25$^+$FoxP3$^+$ T regulatory (Treg) cells. The red arrow denotes drill-down gating strategy. (**C**) Representative FACS plots of CD25 and FoxP3 staining using the gating strategy in (B) at day 7 after conditioning and BMT in the colon of a BALB/c recipient of allogeneic BMT (C57BL/6 →BALB/c). Percentage of cells in each gate is given within the gate. *WT:* wild type; *TBI:* total body irradiation; *BM:* 10 x 10^6 CD45.1$^+$ congenic C57BL/6 or BALB/c donor bone marrow cells; *Teff:* T effector cells; *Treg:* Foxp3$^+$ T regulatory cells.

Figure 3: Non-myeloablative TLI/ATS but not TBI/ATS conditioning decreases donor TCRαβ$^+$CD8$^+$ effector T cell accumulation. (**A**) Representative FACS plots of CD4 and CD8 staining of gated H-2K^{b+}TCRαβ$^+$ cells from donor H-2K^{b+} C57BL/6 mice in spleen (top row), mesenteric lymph node (MLN) (middle row), and colon (bottom row) of recipients at day 6 after conditioning and transplantation. Percentage of cells in each gate is given above the gate. (**B**) Mean ± SEM absolute number (log 10) H-2K^{b+}TCRαβ$^+$CD8$^+$ cells in spleen (top panel), MLN (middle panel), and colon (bottom panel) of recipients at day 6 after conditioning and BMT. *WT:* wild-type; *TBI:* total body irradiation; *TLI:* total lymphoid irradiation; *ATS:* anti-thymocyte serum; *BM:* 50 x 10^6 WT C57BL/6 donor bone marrow cells; *SPL:* 60 x 10^6 WT C57BL/6 donor spleen cells; *TBI800, TBI400*: cGy doses of myeloablative (TBI800) or non-myeloablative (TBI400) TBI. *This figure has been modified from van der Merwe et al[20].Copyright 2013. The American Association of Immunologists, Inc.

Table 1: Solution Preparation Table

DISCUSSION:

This visual protocol describes well-tolerated methods for the isolation of colonic mononuclear cells including lamina propria lymphocytes (LPL). Given that this protocol was optimized in evaluating severe post-transplant mouse colitis models where inflammatory cytokines and tissue injury lend themselves to poor viability of recovered MNC, we anticipate that these methods can be translated to other applications requiring phenotypic analysis of colonic MNC. These include but, are not limited to assessing colon inflammation in mouse models of inflammatory bowel disease, studies of immune responses of colitis-targeted treatments, and colitis produced by infectious pathogens. Additionally, our data using isolations in healthy (syngeneic BMT) mice indicate that the isolation procedure does not require significant inflammatory infiltrate to allow immune cell detection in the MNC isolates. Indeed, similar data have been obtained using untreated healthy (non-BMT) mice (data not shown).

Several key steps of this protocol differentiate it from other published methods and contribute to high yield and viability. For instance, optimization of *Clostridium histolyticum*-derived collagenase E activity (100 U/mL final activity level) allows consistent calculations for different lots of enzyme over long-range experiments[26]. There are 28 different members in the collagen family, together constituting nearly 30% of all proteins in the mammalian body[27]. In addition, different tissues have distinct distributions of collagen subtypes, each requiring unique collagenases for digestion[28]. Collagenase from *C. histolyticum* includes 6 different proteins classified into two classes[29,30]. The specific type of collagenase used can alter the viability and overall quality of the cells isolated from the colon[31]. Previous studies have demonstrated that collagenase type C-2139 (Collagenase E) allows a high yield of lymphocytes among MNC isolated from the small intestine[25]. However, these protocols did not address digestion of colon, a much more muscular organ with significantly more complex collagen composition than the small intestine.

Adequacy and dependability of enzymatic tissue degradation is an established factor influencing overall cell yield and viability by minimizing the need for recurrent mechanical disruption (which induces increased mechanical trauma to the tissue). Due to adequate enzymatic digestion of the interstitial and mucosal collagen using a collagenase E-specific protocol (as compared to collagenase D-mediated digestion protocols in standard use), this protocol minimizes the amount of mechanical manipulation of the tissue fragments required to disrupt the interstitium and release immune cell subsets into suspension. This further enhances the viability of isolated MNC for subsequent assays. As demonstrated in the data (**Figure 1B**), this eliminates the need for DNAse and other chemical or mechanical anti-clumping maneuvers beyond standard filtration of a single cell suspension through a strainer. Other reports outlining the isolation of intestinal lymphoid cells have used dithiothreitol (DTT) and EDTA as mucolytic agents to enhance both yield and viability of the isolated mononuclear leukocytes[24].

Another unique aspect of this protocol which improves cell yield is the application of a fine-tuned silica-based density separation media gradient. Other digestion methods papers published to date do not use such a density separation gradient[21,31]. However, in the authors' experience and those of collaborators utilizing this protocol to isolate functionally active

lymphoid cells, density gradient purification improves both the viability and the purity of MNC recovered following digestion[19,20, 32].

Though not a focus of this manuscript, worthy of mention for those working with intestinal inflammation models in mice is that the methods in this manuscript can be modified to allow similar high-quality isolation of viable MNC from the small intestine. The modification of the primary colonic protocol required to achieve this is the use of a single 90-min digestion (rather than two 60-min digestions), with all other steps (including enzyme activity level and quench steps) identical to those shown. This modification typically yields a range of 0.8 - 4 x 10^6 MNC per small intestine without or 1.5-2.5 x 10^6 MNC with the inclusion of the terminal ileum including the leukocyte-rich cecal cap. Therefore, one novelty of this protocol is that applying the primary protocol to the colon and the modified protocol to the small intestine, one could isolate MNC with high reproducibility and good viability from both small intestine and colon of individual experimental animals in the same experiment.

In summary, the protocol described allows efficient and reproducible isolation of mononuclear cells from the colon or, with modifications, the small intestine. With 2 proficient operators working together, as many as 10 separate colons can be processed and analyzed on a single day, and single-cell suspensions can be ready for subsequent phenotypic and functional analysis within 6-8 h from tissue harvest. Application of this protocol may prove valuable for other research aims needing immune assessment of colonic inflammation, allowing other investigators to characterize the immune system of the mouse colon in a rigorous and reproducible manner.

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DISCLOSURES:
The authors declare no competing financial interests.

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Figure 1

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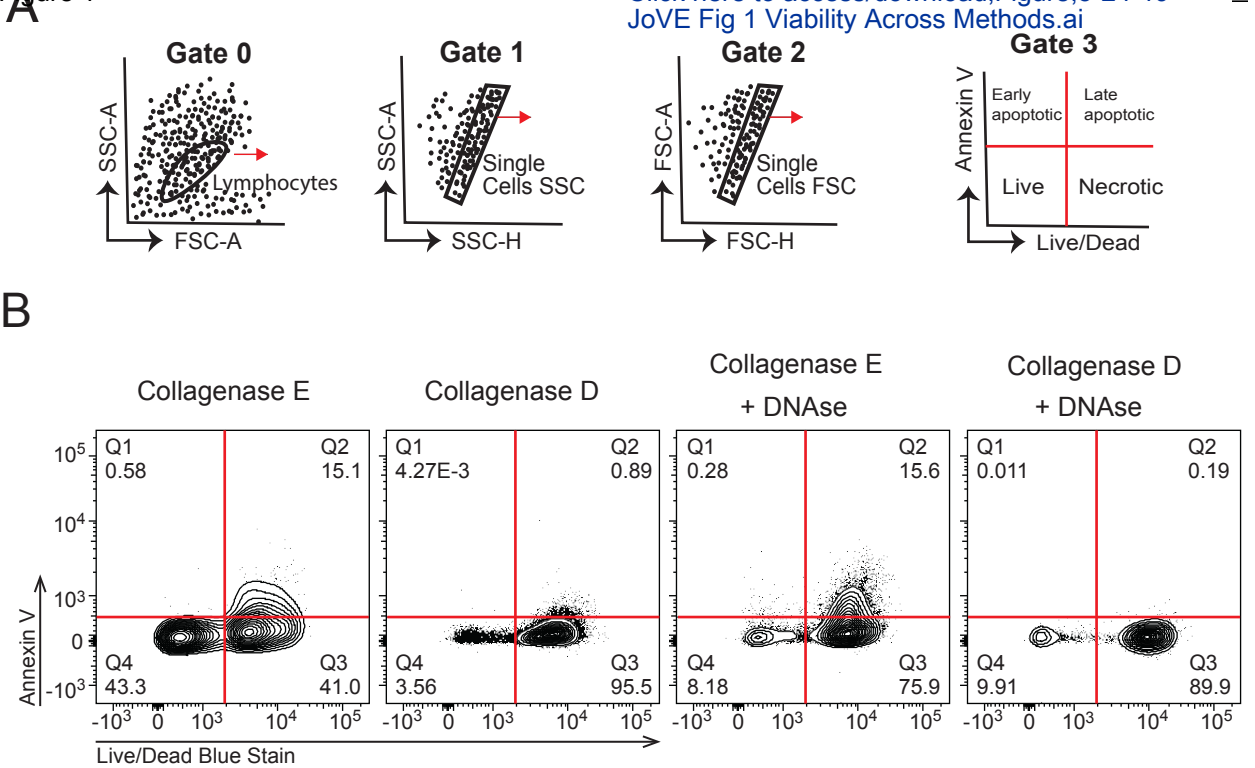

Figure 2

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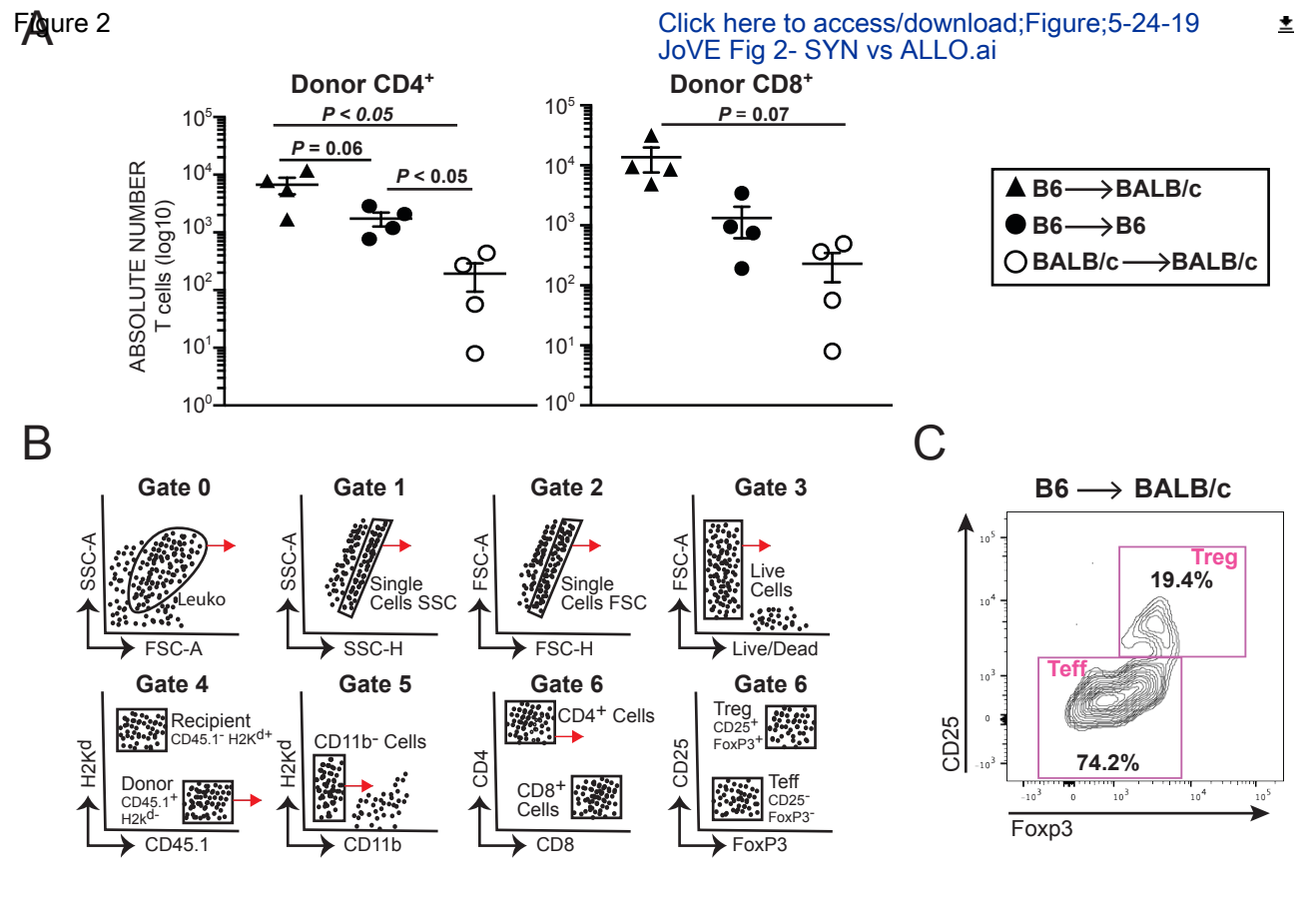

Figure 2

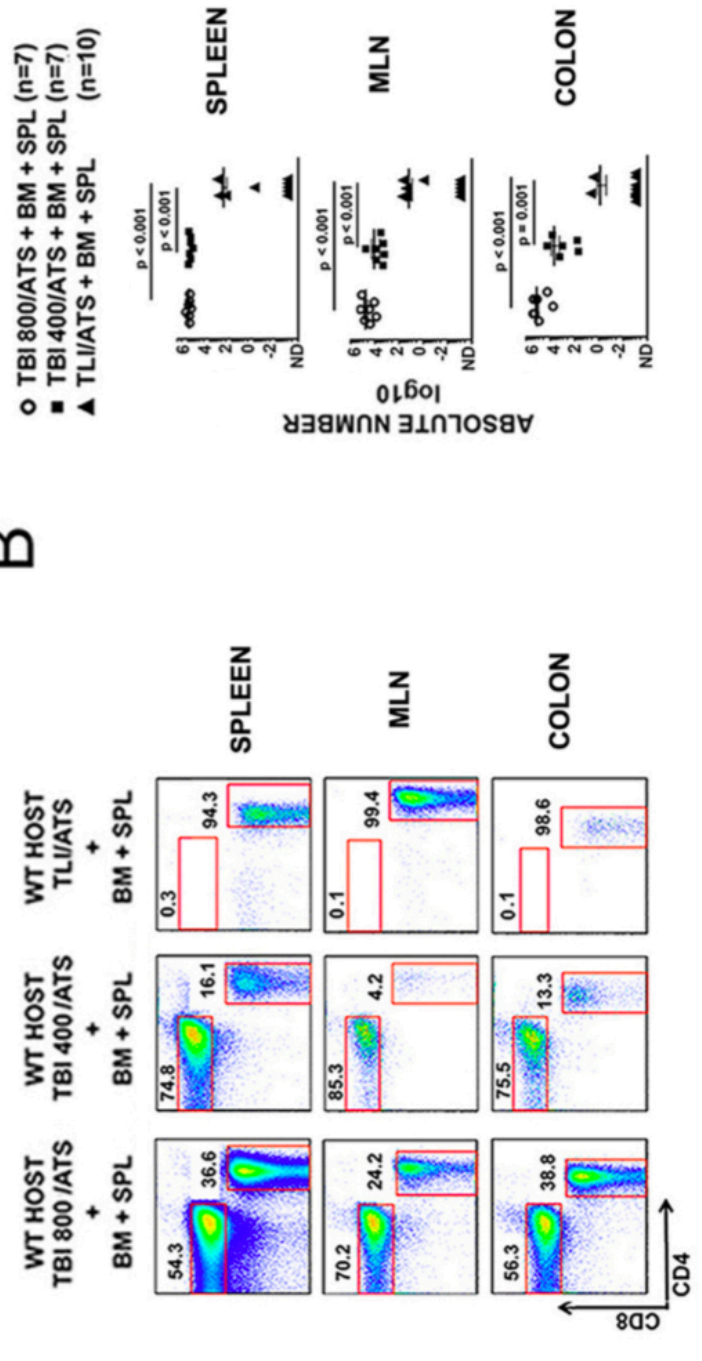

Table 1

Solution	Formula
Colon Buffer	500 mL RPMI + 10mM HEPES + 10% FBS (heat-inactivated at 56°C for 60 minutes, pH adjusted to 7.3)
Silica-Based Density Gradient Media 100% (per colon)	22.5 mL of **Silica-Based Density Gradient Media** + 2.5 mL of 10x PBS.
Silica-Based Density Gradient Media 66% (per colon)	10.72 mL **Silica-Based Density Gradient Media** 100% + 5.28 mL **Colon Buffer**
Silica-Based Density Gradient Media 44% (per colon**)**	11 mL **Silica-Based Density Gradient Media** 100% + 14 mL **Colon Buffer**
Collagenase Digestion Buffer (per colon)	100 U/mL of Collagenase E from *Clostridium histolyticum*, dissolved in 40 mL **Colon Buffer**
FACS Buffer	500 mL 1x PBS + 5 g BSA + 1 mm EDTA + 0.2 g Sodium Azide

Name of Material/ Equipment	Company	Catalog Number
60 mm Petri DIsh	Thermo Scientific	150288
1x PBS	Corning	21-040-CV
10x PBS	Lonza BioWhittaker	BW17-517Q
10 mL Disposable Serological Pipette	Corning	4100
10mL Syringe	Becton Dickinson	302995
15mL Non-Sterile Conical Tubes	TruLine	TR2002
18- gauge Blunt Needle	Becton Dickinson	305180
25 mL Disposable Serological Pipette	Corning	4250
40 micrometer pore size Cell Strainer	Corning	352340
50 mL Falcon Tube	Corning	21008-951
Bovine Serum Albumin (BSA)	Sigma	A4503-1KG
Fixation Buffer	Biolegend	420801
E. coli Collagenase E from Clostridium histolyticum	Sigma	C2139
EDTA, 0.5M Sterile Solution	Amresco	E177-500ML
Fetal Bovine Serum	Thermo /Fisher Scientific - HyCLone	SV30014.03
HEPES	GE Healthcare-HyClone	SH30237.01

Percoll	GE Healthcare-Life Sciences	1708901
RPMI Medium	Corning	17-105-CV
Sodium Azide	VWR Life Science Amresco	97064-646
Trypan Blue	Lonza BioWhittaker	17-942E

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Author(s):	Duneia McManus, Horacio J. Novaira PhD, Sunil Joshi DVM,PhD, Anouk A.J. Hamers PhD, Asha B. Pillai, MD

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RESPONSE TO EDITOR AND REVIEWERS:

We thank both the editor and reviewers for their thorough and helpful comments, which have significantly improved the quality of the manuscript. This is our point-by-point response to comments and the corresponding modifications introduced in the revised manuscript.

EDITOR

Editorial comments:
Changes to be made by the Author(s):

Comment 1:
Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
Response 1:
We have thoroughly proof-read and edited to address the above.

Comment 2:
Please define all abbreviations during the first-time use.
Response 2:
We have thoroughly edited all working parts of the manuscript to ensure that all abbreviations are defined during the first-time use.

Comment 3:
Please revise the Introduction to include all of the following:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application.
Response 3:
We thank the editor for this critique. We have thoroughly restructured the introduction to address the above suggestions. We have taken heed to make the introduction less focused on graft-versus-host disease and of broader relevance to the readership of JoVE. Overall goals and rationale (**lines 61-81**), advantages over and context relative to existing/published methods (**lines 105-112**), and information highlighting the specific applications of this method (**lines 110-112**) have been emphasized to provide context to the readership.

Comment 4:
Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.
Response 4:
We have included this ethics statement above the Protocol section (**lines 114-117**) of the revised manuscript.

Comment 5:

JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example, Falcon, Nitex, Percoll, etc.

Response 5:

We have removed all commercial language from the new manuscript. We have substituted commercial brands with the following:

Falcon tube →polypropylene conical tube.

Nitex filter → filtration fabric cell strainer.

Percoll→Silica-Based Density Separation Media/gradient.

Comment 6:

Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly.

Response 6:

We have edited all working parts of the protocol to ensure that each step starts in the imperative tense. "NOTE" is now used sparingly throughout the protocol, only where necessary to bring attention to key points where protocol error can occur if not emphasized.

Comment 7:

Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.

Response 7:

We have edited to ensure that the numbering follows the JoVe Instructions for Authors.

Comment 8:

Please make subheadings and then steps in the protocol section.

Response 8:

We have included subheadings to conceptually organize steps in the protocol section.

Comment 9:

The Protocol should contain only action items in complete sentences that direct the reader to do something.

Response 9:

Only action items in complete sentences have been included. Conciseness was prioritized.

Comment 10:

The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please ensure that individual steps in the protocol contain only 2-3 actions per step and a maximum of 4 sentences per step.

Response 10:

We have edited heavily for conciseness and active tense, with the goal that sections are discrete and easy to follow.

Comment 11:
Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).
Response 11:
Personal pronouns have been removed in this revision.

Comment 12:
Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?
Response 12:
In revising the manuscript, we have paid attention to provided precise details in each step such that the reader should be able to visualize the protocol steps succinctly.

Comment 13:
Step 1: Please write in complete sentences or make a table in .xlsx format and upload it to the editorial manager account separately. Refer the table wherever required.
Response 13:
We thank the editor for this helpful organizational guidance. We have created a table as a .xlsx file entitled "Solutions Preparation Table".

We refer the reader to this table in **lines 120 to 124**:

> 1.1 Mix and prepare the **Colon Buffer**, **Silica-Based Density Separation Media 100%, Silica-Based Density Separation Media 66%**, **Silica-Based Density Separation Media 44%**, **Collagenase Digestion Buffer** and **FACS Buffer as described in the Solutions Preparation Table.**

Comment 14:
2.1.1: Do you clean the area with ethanol? Do you remove the fur? Please write all the steps
Response 14:
We have clarified these points in revised steps 2.2-2.3 (**lines 145-152)**.

Comment 15:
2.1.2: Do you first perform the incision in the skin? Please write all actions
Response 15:
We have clarified these points in revised steps 2.2-2.3 **(lines 145-152**).

Comment 16:
Please use the symbol µ instead of u for micron throughout the manuscript.
Response 16:
We have utilized symbol "µ" in place of "u" for "micron", throughout.

Comment 17:
13-18: Please explain the gating strategy for FACS.
Response17:
We have included in Figures 1 and 2 a visual outline of the step-by-step flow cytometry gating strategy used for each set of data shown.

Comment 18:
There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Response 18:
The most essential protocol steps for video filming have been highlighted **green** in the attached revision.

Comment 19:
Please describe the result with respect to your experiment, you performed an experiment, how did it helped you to conclude what you wanted to and how is it in line with the title.
Response 19:
We have made the following changes to the representative results section so that we are appropriately describing the result with respect to.

Comment 20:
The protocol ends at trypan blue staining. Please provide the result for the same.
Response 20:
We thank the editor for this suggestion. In response to this request and that of another reviewer, we originally performed studies with both Trypan blue staining and with flow cytometry. This data revealed the flow cytometry was much more sensitive for detecting differences in viability between mononuclear leukocytes isolated between differing protocols. Therefore, we have elected to show details of the more sensitive comparison (**Figure 1).** This shows representative images and a graphical representation of Annexin V (apoptosis) versus Live/Dead Blue (necrosis) staining for the evaluation of cell death when the protocol uses either Collagenase E or D with or without DNAse 1. Median values for key parameters represented in this figure are also included in the Results section.

Comment 21:
How do you figure out that the cells isolated are actually Lamina Propria? Do you perform any marker studies? Please include protocol and the results for the same.
Response 21:
We are unaware of any useful marker that dependably differentiates LP lymphocytes from lymphocytes of other origin in the colon. We have removed reference to LPL throughout the manuscript, rewording this more accurately as “mononuclear cells” (MNC).

Comment 22:
Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”
Response 22:
The American Association of Immunologists, Inc., has granted permission to reproduce Figure 1 from that manuscript (Figure 3 in the current JoVE manuscript) from the article “Recipient Myeloid-derived Immunomodulatory Cells Induce PD-1 Ligand-Dependent Donor CD4+Foxp3+ Treg Proliferation and Donor-Recipient Immune Tolerance After Murine Non- myeloablative Bone Marrow Transplantation”. We have uploaded the relevant documentation of explicit permission with the resubmission documents.

Comment 23:
As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Response 23:
We thank the editor for this critique. We have thoroughly revised the Discussion to include all of the points listed.

Comment 24:
Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

Response 24:
We have removed all tables from the manuscript and have uploaded appropriately.

Comment 25:
Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).] For more than 6 authors, list only the first author then et al.

Response 25:
We have edited all references to ensure they are in compliance with JoVE's guidelines for citations and references.

REVIEWER #1

Reviewer #1 Comment 1:
The manuscript describes a method for the isolation of LPMC from murine colon. The presented protocol is clear described, but this is all and the manuscript raises some critical questions.

Not mentioned is the clumping by the authors after the collagenase digestion step.
DNase I is not included in the protocol as this also reduces cell clumping.
The authors announced in the abstract that by this protocol a significant number of viable lymphocytes could be isolated, but no figures or data to underline the viability is shown. How is the survival rate of LPMC's from their protocol? What about apoptotic and necrotic cells?
The novelty character of the presented protocol is less as there exist several manuscripts since last century, which describes the steps better. Not even the key papers in this field are mentioned like Bull et al. 1977 JCI or Davies et al. 1981 GUT.
Response 1:
We have conducted a widespread literary search to be able to report the trypsin activity and any other data to pertaining to Collagenase E, Type VIII, especially in comparison to Collagenase D. However, search results were not successful in locating such information. We have chosen to address your concerns about the use of Collagenase E in comparison to Collagenase D with and without DNAse 1 in an experiment to compare the output result. Representative results are provided in **Figure 1** and cumulative findings on necrosis discussed in the Results section of the manuscript.

We have also chosen to cite the key papers in the field as mentioned, in both the Introduction and in the Discussion (where we compare the current protocol to those published methods to point out salient differences).

REVIEWER #2

Reviewer #2 Comment 1

Manuscript Summary:

This is a well described procedure to isolate LP cells from the colon. As seen from the results, there is a good yield of cells from the colon. I have a few minor comments that will improve the readability of the manuscript

Major Concerns:

none

Minor Concerns:

Comment 1:

The first half of the abstract (and introduction) talks about transplantation, allo-HCST, engraftment etc., which is not relevant to the question addressed in this paper. The abstract should be kept general and discuss the importance of isolating cells from different regions of the intestine. For example, small intestine is well studied but no methodology exists to isolate cells from the colon. The relevant details about the stem cell transplantation and GVHD etc can be mentioned in the results sections, just enough details to help the readers understand the FACS data and the context. I suggest these sections be edited. The discussion is well written and very clear. Maintain the same tone for the intro and results.

Response 1:

We thank the Reviewer #2 for this critique. We have thoroughly restructured the introduction and abstract to include all suggestions made.

We have kept our description of allo-HSCT in the results and discussion and minimized such in the introduction. In the introduction we have focused on applications of this protocol to study other sources of gut inflammation in the gut to convey an overarching sense of what models are suitable for the application of the proposed methods.

Comment 2:

To be consistent, on line 212 what is the recipe for your FACS Buffer? I would suggest mention that on line 114 along with the other solutions.

Response 2:

We thank Reviewer #2 for this critique. We have mentioned the FACS Buffer on **line 123**. We have also uploaded a table in the .xlsx file entitled **"Solutions Preparation Table"**, which includes the recipe for FACS buffer.

Comment 3:

Will this protocol also work for isolation of cells from the small intestine? If so, this should be mentioned in the results or discussion.

Response 3:

We thank Reviewer #2 for this particular question, which helps to clarify the novelty of the protocol in a way we had not considered presenting earlier. Yes, this protocol will work for the isolation of cells from the small intestine, but with modification. This is a uniqueness of the protocol in that, using the same reagents, one could potentially isolate MNC reproducibly and with good viability from both the small and the large intestines of the same animal on the same day. This allows simultaneous study of MNCs isolated from both organs using one isolation effort. We have referenced this novelty in the introduction (**lines 96-98**), specific protocol modifications in the discussion (**lines 472-475**), and relevance to murine gastrointestinal model research (**lines 477-4780**).

REVIEWER #3

The method describes how to isolate lymphocytes from colonic lamina propria in mice. The steps listed in the procedure are clearly explained and the protocol should lead to the described outcome. However, some minor points need to be improved:

Comment 1:
The title should indicate that the method allows isolation of mononuclear cells including lymphocytes.
Response 1:
We thank Reviewer #3 for this critique of the title. We have updated the title accordingly.

Comment 2:
In the abstract, "auto-immune conditions" should be removed since Crohn's disease and Ulcerative Colitis are not autoimmune diseases as usually defined by immunologists (lines 105-106 in the text).
Response 2:
We have removed "auto-immune conditions" from the text when describing Crohn's disease and Ulcerative Colitis. We have instead referred to these conditions as inflammatory bowel diseases.

Comment 3:
In the figure 1, it would be useful to mention in the legend that FACs analysis has been performed on mononuclear cells isolated from colon of only one mouse.
Response 3:
Wherever possible in all figure legends including **Figure 1**, we have clarified that representative plots or data are from N =1 mouse.

Comment 4:
To my opinion the legend of the **Figure 2** should be improved to be more intelligible for the readers, even if this is a technical analysis. For example, the authors should indicate that donor C57BL/6 mouse splenocytes are H2Kb+, explain what total lymphoid irradiation is and in this latter case, why only CD4+ T lymphocytes are present after transplantation etc.
Response 4:
We thank Reviewer #3 for bringing this lack of clarity to our attention. We have edited the figure legend to explicitly state that C57BL/6 mice are H-2K^{b+}. In the beginning of the representative results section, we have also explained the difference between TBI and TLI and elaborated why the CD8^{+} T cells accumulation is decreased (significance of the results), etc, so that the reader is given better context.
Some specific edited regions may be reviewed as follows:
Lines 390-393:

> "**(A) Representative FACS plots of CD4 and CD8 staining of gated H-2K^{b+}TCRαβ^{+} cells from donor H-2K^{b+} C57BL/6 mice** in spleen (*top row*), mesenteric lymph node (MLN) (*middle row*), and colon (*bottom row*) of recipients at day 6 after conditioning and transplantation. Percentage of cells in each gate is given above the gate."

Lines 3150-325:

> "**Figure 3** shows an extended application of this method in historic data from our group using the presented protocol to compare accumulation of GVHD-inducing CD8+ versus CD4+ donor-derived T cells in the colon of BALB/c mice either protected or not protected from GVHD by the pre-BMT treatment preparative (conditioning) regimen.[12] The tested preparative regimens included 800 cGy/myeloablative total body irradiation (TBI800) or non-myeloablative TBI

(400TBI), as well as nonmyeloablative conditioning using total lymphoid irradiation (TLI) in which irradiation was delivered to the lymph nodes, thymus, and spleen with shielding of the skull, lungs, limbs, pelvis and tail. All conditioning was combined with anti-thymocyte serum (ATS), an immunomodulating agent. As early as day 6 after BMT, this colonic MNC isolation protocol resulted in robust flow cytometric analyses as compared to identical analyses on more lymphocyte-enriched GVHD target organs such as spleen and mesenteric lymph nodes (MLN) (**Figure 3A).**[20] Reproducible isolation of colonic MNC across BMT recipients (n = 7-10 per treatment group) allowed for a robust statistical comparison of absolute numbers of donor $CD8^+$ effector T cells between different pre-transplant conditioning treatment groups **(Figure 3B)**, yielding important data on immune phenotypes that led to key studies revealing the innate immune mechanisms of GVHD protection from TLI as opposed to TBI pre-BMT conditioning.[20]"

Comment 5:

Finally, it would be useful to also indicate the absolute number of T cells that can be recovered in the small intestine to compare it with the colon.

Comment 5:

This protocol can also be applied for the isolation of cells from the small intestine, but with modification. In the discussion portion, we have referenced this particular modification with estimated absolute number of T cells that can be recovered in the small intestine (**lines 474-476**).

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May 16, 2019

Dr. Asha Pillai
University of Miami -Batchelor Children's Research
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Email: asha.pillai@miami.edu

Dear Dr. Pillai:

The American Association of Immunologists, Inc., grants permission to reproduce Figure 1 from the article "Recipient Myeloid-derived Immunomodulatory Cells Induce PD-1 Ligand-Dependent Donor CD4+Foxp3+ Treg Proliferation and Donor-Recipient Immune Tolerance After Murine Non- myeloablative Bone Marrow Transplantation" published in *The Journal of Immunology*, vol. 191, pp. 1-13, 2013, in the "*Efficient Isolation of Lamina Propria Lymphocytes from Murine Colon*," published by JoVE - Journal of Visualized Experiments, contingent on the following conditions:

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Sincerely,

Catherine Wattenberg
Director of Publications
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